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Temporal Determinants of Neonatal Alcohol-Induced Cerebellar Damage and Motor Performance Deficits

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GOODLETT, C. R. AND K. R. LUNDAHL. *Temporal determinants of neonatal alcohol-induced cerebellar damage and motor performance deficits.* PHARMACOL BIOCHEM BEHAV **55**(4) 531–540, 1996.—The timing and duration of alcohol exposure was manipulated in neonatal rats by using a "binge" model of alcohol exposure during the "third trimester equivalent." Groups of Sprague-Dawley rats were exposed to binges via artificial rearing on postnatal days (PD) 4–9, on PD 4–6 or on PD 7–9, which produced peak blood alcohol concentrations representative of human alcohol abusers (~250 mg/dl). Motor performance was assessed using parallel bar traversal on PD 42–44, and total Purkinje cell numbers were determined by using the 3-dimensional stereological optical fractionator method. PD 4–9 binge exposure induced the most severe Purkinje cell loss (to 68% of controls) and PD 4–6 binge exposure also produced significant loss (to 86% of controls), whereas PD 7–9 binge exposure had no significant effect (98% of controls). Unexpectedly, all three alcohol treatments resulted in significant impairments on the parallel bar task. The time of exposure during the early neonatal period in rats strongly influences the degree of Purkinje cell loss, but Purkinje cell loss is not necessary for the alcohol-induced motor performance deficits. Both neuromorphological and neurobehavioral assessments are needed for a full description of alcohol-related neurodevelopmental disorders. **Copyright © 1996 Elsevier Science Inc.**

Alcohol Cerebellum Purkinje cells Motor performance Fetal alcohol syndrome Neonatal

SOME of the most commonly reported outcomes of human fetal alcohol exposure are impaired motor coordination, impaired motor skills and gait abnormalities (2,24). Human neuropathological studies (9) and recent neuroimaging studies (26,32) in children identified as having fetal alcohol syndrome (FAS) have also shown that structural deficits of the cerebellum are common outcomes in FAS. These findings suggest that the developing human cerebellum is particularly vulnerable to alcohol-induced damage and that motor impairments in human FAS may in part reflect functional consequences of such damage.

Experimental findings using animal models support these observations and suggest that the period of cerebellar development comparable to that of the human third trimester may be a period particularly vulnerable to alcohol. Many of the brain developmental events comparable to those that transpire over the last 15 weeks of human fetal development occur in the rat during the neonatal "brain growth spurt" (10). Most pertinent, human Purkinje cell dendritic outgrowth is initiated around fetal week 24 and continues through the third trimester (38). In rats, comparable changes in Purkinje cell dendritic morphology occur between postnatal days (PD) 4–12 (1,4,13). Numerous studies have shown that alcohol exposure during the first 2 postnatal weeks produces permanent cerebellar growth restriction and reductions in Purkinje and granule cell density and/or total neuron number, particularly when the alcohol exposure occurs in a bingelike pattern (3,6,7,12,25,29, 30). Reductions in Purkinje cell density produced by alcohol exposure during Purkinje cell differentiation is significantly more severe than comparable exposure during the prenatal period of Purkinje cell neurogenesis (25). Neonatal alcohol exposure also impairs later motor function as indicated by altered gait (28) and deficits in motor performance (17,29,34). In summary, alcohol exposure during the period of Purkinje cell differentiation has severe long-term structural and functional consequences.

Recent studies in rats have demonstrated that there are striking temporal windows of enhanced vulnerability of the

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neonatal cerebellum to structural damage by ethanol, with the time of greatest vulnerability occurring in the last half of the first postnatal week. Heavy binge exposure given 1 (12) or 2 (22,33) days during the first postnatal week induced significantly more severe reductions in Purkinje cell density and total cell number than similar exposure just a few days later (during the second postnatal week). Each of these studies used a heavy bingelike exposure (6.6 g/kg/day, given via artificial rearing as a 15% v/v solution in milk formula in two feedings 2 h apart) to produce mean peak blood alcohol concentrations (BACs) greater than 350 mg/dl. When given on PD 4 (or PD 4 and 5), these high-peak BACs produced massive reductions in total Purkinje cell numbers (to 55–60% of control numbers), whereas comparable exposure on PD 8 and/or PD 9 resulted in significantly less severe Purkinje cell loss (88–94% of controls).

These findings indicate that the last half of the first postnatal week in rats, marked by the onset of dendritic development of Purkinje cells, is part of a temporal window of vulnerability to alcohol-induced Purkinje cell loss. The purpose of the current study was threefold. The first goal was to use bingelike neonatal alcohol treatments that produce BACs that are more representative (than the previously cited studies) of BACs found in human alcohol abusers to test the hypothesis that exposure throughout this period (PD 4–9) will produce significant Purkinje cell loss and motor performance impairments. The second goal was to test the hypothesis that binge exposure during the end of the first postnatal week (PD 4–6) will produce more severe Purkinje cell loss and more severe motor performance deficits than comparable exposure during the beginning of the second postnatal week (PD 7–9). The third goal was to test the hypothesis that Purkinje cell loss and motor performance deficits produced by binge exposure throughout this period (PD 4–9) will be more severe than that produced by either 3-day exposure (PD 4–6 or PD 7–9).

Alcohol was administered during the neonatal period by using artificial rearing methods that have been well established for developmental alcohol studies (11,36). The total number of Purkinje cells in the cerebellum was estimated by using the unbiased, 3-dimensional, stereological optical fractionator method (19,20,37). Motor performance was assessed at PD 42 by using parallel bar traversal, a task sensitive to experimental cerebellar damage (8) and used previously to demonstrate motor performance deficits following neonatal alcohol exposure (29,34).

METHOD

Subjects

One hundred three rats from 16 litters resulting from timed pregnancies of Harlan Sprague-Dawley dams bred in the IUPUI vivarium were used in this study. Gestational day 0 was identified by the presence of sperm in a vaginal smear taken the morning after an overnight mating. Litters were culled to 10 pups (5 males, 5 females whenever possible) on the day after birth. The day of birth was nearly always gestational day 22. The developmental timing of all treatments was based on gestational age; reference to postnatal ages (as a convenience) considers gestational day 22 as the day of birth (PD 0). The breeders, their suckling litters and the weaned rats were maintained in the IUPUI vivarium with ad libitum food and water on a 12 h:12 h light:dark cycle with lights on at 0700 hours. Offspring were weaned at 21 days of age and housed 2–4 per cage with same-sex littermates thereafter. The vivarium was maintained at 22°C ± 2°.

All of the subjects were tested on PD 26–31 on Morris maze swim-escape place learning, and those results along with growth curves, BACs and perfused whole brain weights have been published elsewhere (16). The data concerning parallel bar performance and cerebellar Purkinje cell counts have been presented in abstract form (15,23).

Groups

The experiment used the following five treatment groups (PD 4 = gestational day 26): alcohol exposure on PD 4 through PD 9 (designated PD 4–9), alcohol exposure on PD 4 through PD 6 (PD 4–6), alcohol exposure on PD 7 through PD 9 (PD 7–9), an artificially reared gastrostomy control group (GC) and a normally reared suckle control group (SC). On gestational day 26, the rats were assigned within litter to the five treatment groups, one male and one female to each group. The final group numbers are given in Table 1.

Artificial Rearing

The rats assigned to the artificial rearing groups were surgically implanted with intragastric feeding tubes under methoxyflurane (Pitman-Moore, Mundelein, IL) anesthesia on PD 4 [see (6,14,31,36) for detailed descriptions]. Briefly, a sterile PE-10 tube with a small flexible washer secured to one end was attached to a stainless steel rod covered with Silastic®, passed down the esophagus and externalized through the stomach and abdominal walls. The tube was secured against the abdomen with another press-fit washer and threaded through the nape of the neck.

The pups were fed a customized milk formula (36) through the intragastric tubes by using timer-controlled Harvard Model 22 infusion pumps. Each day, the rats were fed a volume of formula (in milliliters) equal to 33% of the mean body weight (in grams) of the litter being reared. The pups were weighed each morning, and excretion was stimulated at least twice a day by gentle anogenital stroking. For the alcohol groups, on the morning of the designated exposure days the pups were given two consecutive feedings of a 10.2% (v/v) solution of alcohol in milk formula, yielding a daily alcohol dose of 4.5 g/kg. For the GC group and for the alcohol groups on nonexposure days, the two daily feedings matched to the alcohol feeding used an alcohol-free milk formula solution made isocaloric by the addition of maltose-dextrin. On the remaining 10 feedings each day and throughout PD 10 and PD 11, all groups were given milk formula alone. On PD 11 and PD 12, the pups were also given opportunities to lick milk formula from an artificial nipple, a procedure that assists their transition back to suckling. On PD 12, the pups were given a number code by injection of a small amount of India ink into one or more of the paws, the intragastric tubes were cut and heat sealed, and the pups were painted with a slurry made from the lactating dam's feces from the nest and fostered back to the litter of that dam.

After the second alcohol feeding on PD 6 and on PD 9, a 20-μl sample of blood was collected in heparinized capillary tubes from a tail-clip of each artificially reared pup. The blood was placed immediately into 1.3 ml of trichloroacetic acid (6.25% w/v) in 1.5-ml microcentrifuge tubes, sealed tightly and stored at 4°C, and assayed with a enzymatic assay for ethanol content (Kit 332-BT, Sigma, St. Louis, MO) by using a Guilford Response™ spectrophotometer. The concentration of alcohol in each sample, expressed as milligrams ethanol

TABLE 1

TREATMENT CHARACTERISTICS AND EFFECTS ON BODY WEIGHT AND CEREBELLUM WEIGHT AT THE AGE OF DEATH (PD 45)

Group	*n*	Mean peak BAC (mg/dl)†	Body weight (g)	Cerebellum weight (mg)	CB-to-body weight ratio (mg/g)
Females					
SC	10	—	160 ± 4	202 ± 4	1.27 ± .02
GC	10	—	148 ± 4	187 ± 3	1.27 ± .03
PD 4–6	9	242 ± 18	148 ± 3	170 ± 5	1.15 ± .03
PD 7–9	9	238 ± 14	154 ± 3	187 ± 4	1.24 ± .03
PD 4–9	11	265 ± 17	153 ± 4	162 ± 2	1.08 ± .02
Males					
SC	13	—	200 ± 5	215 ± 4	1.08 ± .02
GC	10	—	185 ± 2	199 ± 5	1.08 ± .02
PD 4–6	10	230 ± 20	187 ± 5	188 ± 3	1.01 ± .02
PD 7–9	9	241 ± 10	181 ± 3	194 ± 3	1.08 ± .02
PD 4–9	12	267 ± 10	185 ± 3	168 ± 8	0.91 ± .03

The SC group had heavier body weights and absolute cerebellar weights than did the artificially reared groups, but the PD 4–9 and the PD 4–6 alcohol treatments reduced cerebellar weight (but not body weight) compared with the gastrostomy control group. The significant main effects of group and sex are described in the Results section.

†Peak blood alcohol concentrations (BACs) were determined on PD 6 for the PD 4–6 group and on PD 9 for the PD 4–9 group and are the average for the two determinations (PD 6 and PD 9) for the PD 4–9 group. These two determinations for the PD 4–9 group were not significantly different from each other (16).

per 100 ml of blood (mg/dl), was determined by comparing the absorbance (at 340 nm) of the sample to a concurrently derived standard curve of five known alcohol concentrations (0–450 mg/dl).

Parallel Bar Testing

The apparatus consisted of two parallel steel rods (each 0.48 cm in diameter, 61 cm long) held between plywood end platforms (15.3 × 17.8 cm). The rods were fastened with screws onto a rack of 28 grooved slots (0.5 cm apart) on each platform and stood 63 cm above a floor of wood chip bedding. The subjects were tested by an experimenter blind to neonatal treatment condition.

On the first day of testing (PD 42), the subject was initially placed on each platform for 30 s. Then, the subject was carefully placed on the rods halfway between the platforms, with both left paws on one bar and both right paws on the other bar. After stabilizing, the subject was released and usually moved forward toward the escape platform. Four successive alternating steps with the hind legs on the rods constituted a successful traversal. If the subject placed two hind paws on one rod, dropped a hind paw below the rod, stepped twice in succession with the same hind paw, fell or swung under the rods, the trial was considered unsuccessful. If the subject failed to move after 3 min, it was removed, placed in the holding cage for 1 min and then replaced onto the rods, without counting the prior period as a trial. Of the 103 subjects assigned to testing, 8 (4 from the GC group, 3 from the PD 4–9 group and 1 from the PD 7–9 groups) were excluded because they either consistently failed to move after placement on the bars, they consistently reared and attempted to turn around as their first movement, or they attempted fewer than two steps per trial. The final numbers of subjects in each group completing the 3 days of testing are listed in Table 2.

The initial distance between the rods was set at 3.5 cm. Subjects were tested for up to five consecutive trials at a given gap width between rods, with an intertrial interval of 5–10 s. Once successful at a given width (four successive alternating steps with the hind limbs), the subject was placed in a holding cage while the distance between the rods was increased at 0.5-cm increments. If unsuccessful after five consecutive trials, the testing for the day was terminated. Subjects were tested to a maximum of 15 trials a day for 3 consecutive days. Each day, the distance between rods was set at the last successful distance for each individual animal. For each trial, the number of successful steps (up to criterion of four), the latency and the type of error (if present) were recorded. For each day of testing, the following data were recorded: number of successful traversals, number of failures, ratio of successful to total trials, number of steps, maximal gap traversed and mean latency to complete a trial.

Purkinje Cell Counting

Rats were perfused on PD 45 with 0.9% saline followed by 1% (w/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde. After the brains were removed and regional weights (forebrain, cerebellum, brainstem) were taken, the tissues remained in the same fixative for at least 3 weeks. Of the 103 subjects, 5 were not available for histology because they either were not perfused or were inadequately perfused (2 from PD 4–9, 2 from PD 7–9, 1 from GC). A random sample of subjects from each group (8–10/group, approximately equal number of males and females) was taken for Purkinje cell count analysis of the cerebellum.

The optical fractionator used systematic random sampling and unbiased 3-dimensional counting schemes to count Purkinje cells from a known fraction of the entire cerebellum. The total number was calculated by multiplying these sample

TABLE 2

MEASURES OF PERFORMANCE ON THE PARALLEL-BAR MOTOR LEARNING TAS

Group	n	Cumulative number of successful traversals (mean ± SEM)			Daily composite performance score (mean ± SEM)			Daily latencies per trial (in seconds) (mean ± SEM)		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
SC	23	1.3 ± .5	5.5 ± .5	9.2 ± .6	3.1 ± 1.4	12.2 ± 1.7	13.5 ± 2.2	35 ± 5	23 ± 2	20 ± 2
GC	16	1.3 ± .3	5.9 ± .7	9.4 ± .8	2.0 ± 0.6	13.9 ± 2.2	12.8 ± 2.2	31 ± 4	20 ± 3	22 ± 3
PD 4–6	19	1.3 ± .5	4.1* ± .6	7.9 ± .7	2.5 ± 1.1	7.2* ± 1.5	13.5 ± 2.2	49 ± 7	21 ± 2	20 ± 2
PD 7–9	17	0.9 ± .3	3.7* ± .7	6.5* ± .7	1.4 ± 0.6	7.9* ± 1.8	8.1 ± 1.7	32 ± 5	24 ± 7	20 ± 3
PD 4–9	20	0.7 ± .2	3.6* ± .5	6.9* ± .7	1.0 ± 0.3	7.0* ± 1.2	11.9 ± 2.1	42 ± 6	31 ± 5	21 ± 3

For the mathematical definition of the composite performance score, see data analysis in Methods. Most subjects had fewer than two successful traversals on the first training day, but all groups did increase the average number of successful traversals across days. All alcohol-exposed groups had impaired acquisition of the task, which was most apparent on the second training day. Mean latencies to complete the trials did not differ significantly across groups.

*Significantly different ($p < 0.05$) from the control groups (SC and GC combined for LSD comparisons).

counts by the inverse of the sampling fractions taken at each level. The fractionator method, when properly applied, allows unbiased determination of total cell numbers in a 3-dimensional structure without having to determine specifically the "reference" volume containing the cells under investigation (19,20,37).

Frozen sections were cut exhaustively through the entire cerebellum (less the flocculus) in the sagittal plane by using a Reichart-Jung sliding microtome set at a nominal thickness of 55 μm. Beginning with a random start within the first 14 sections, every 14th section was saved, constituting the systematic random sample of sections required by the fractionator method. These were mounted on subbed microscope slides and stained with thionin, and the slides coded with arbitrary numbers.

Three-dimensional counts of Purkinje cells were obtained by an experimenter blind to treatment condition by using the Olympus/Bico Computer-Assisted Stereological Toolbox and a Heidenhain z-axis microcator. A Sony videocamera was attached to a microscope and projected images onto a Sony videoscreen interfaced with an Amiga computer. The screen displayed the image from the microscope overlaid with a graphic counting frame provided by the software. Each section of the complete set for a given cerebellum was sampled in the counts. With the 4× objective and a mouse, the section was outlined. The computer then generated a systematic random sample of locations throughout the tissue section by using a meander program. The meander was a sequence of the computer controlled raster movements of the microscope stage using defined step lengths in the X and Y planes. The locations defined by the meander program that fell within the section constituted the area sampled from that section, from which Purkinje cell counts were made. These locations were a systematic random sample of all possible section locations, and each could be considered a small sample of the larger rectangular patch defined by the X and Y dimensions of the stepper movements. The meander stage movements began at a random starting location just outside of the tissue section, and each movement was effected at the experimenter's convenience by clicking an icon that activated the stage controller to move to the next location on the predetermined meander path.

Three-dimensional counts of Purkinje cells at each location on the meander were obtained by applying the optical disector to obtain an unbiased count of the number of Purkinje cells in an optically defined box within the section of known dimensions (thus, a known volume). The optical box was defined by the X and Y dimensions of the graphic counting frame (149 μm × 109 μm) and in the Z dimension by the measured depth of the z-axis movements (from the microcator) while focusing down through the tissue (6 μm). The tissue was brought into focus with the 60×, 1.4 N.A., oil-immersion objective (plane of focus < 1.0 μm) at each point on the meander. The focal plane was moved 4–5 μm below the top surface of the section, establishing the zero (or exclusion) plane. Any Purkinje cells observed in the exclusion plane, defined by an identifiable intact nuclear membrane observable within the exclusion plane, were not included in the count. The plane of focus was moved down through the tissue to a depth of 6 μm, and Purkinje cells were counted only if they met the inclusion criteria, including at the bottom plane (a plane of inclusion). At each point along the meander in which Purkinje cells were present, counts were taken, and the total number of cells and locations were recorded for each section. The dimensions of the stepper, counting frame and disector height were chosen to yield approximately 150 counts per cerebellum, a strategy that yields the highest efficiency with stereological studies (19,20,37).

Calculation of Total Purkinje Cell Number

The determination of total cell number involved obtaining the sum of all cells counted in the sampled volume, a known fraction of the entire volume of the cerebellum, and multiplying that sum by the inverse of sampling fraction from each of the three sampling levels (section, area, depth). The calculations for a given cerebellum are defined mathematically as follows:

f_1 = sampling fraction for all frozen sections (every 14th section)
= $1/n = 1/14$

f_2 = sampling fraction for each section area (Xstep = 950 μm, Ystep = 900 μm)
= area (counting frame)/area (stepper)
= (16,241 μm^2/(950 μm * 900 μm)

f_3 = sampling fraction for section thickness (t)
= height (disector)/average measured t of stained sections
= (6 μm)/(t)

Equation for estimating total Purkinje cell number in a cerebellum and an example case:

Q^- = sum of Purkinje cell counts from the sampled locations for a given cerebellum

Example: $Q^- = 200$; $t = 18\ \mu m$

$$\text{Total} = Q^- * [1/f_1]\,[1/f_2]\,[1/f_3]$$
$$= 200 * [14/1]\,[(950 * 900)/(16{,}241)]\,[18/6]$$
$$= 4.42 \times 10^5$$

Data Analysis

For the parallel-bar training, several dependent variables were available to measure motor performance, including number of successes, number of failures, number of trials, maximal gap traversed, ratio of successes to total number of trials, number of steps taken and latency to complete the trials. Because most of the performance measures were correlated, the most straightforward measure of the criterion behavior being scored, the cumulative number of successful traversals across days, was chosen a priori as a primary dependent variable. However, because the number of successful traversals was not sensitive to repeated failures at a given gap width before a successful traversal, a second measure was also analyzed, hereafter referred to as the *composite performance score.* This composite score was derived mathematically to reflect both the relative success rate at a given gap (reflected in the ratio of successful to total number of trials at each width) and the increasing degree of difficulty with increasing gaps. A single composite score (C) for each individual was derived for each day by summing the products of the success ratio at each gap attempted multiplied by the width of the gap, defined as follows:

$$C = \sum[(S_i/T_i)(W_i)],$$

where S_i = number of successful trials at a given gap width attempted on a given day (0 or 1), T_i = number of total trials given for that gap width on that day (maximum of 5) and W_i = the width (in centimeters) of the gap for the specific set of trials.

As defined, higher composite scores were attained with fewer failures at a given width, and higher composite scores were also possible as the gap width increased.

For these two measures of motor performance, with mean daily latencies to complete the trials, data were analyzed first with a repeated measures analysis of variance (ANOVA) with group and sex as grouping variables and day as the repeated measure. When significant main or interactive group effects were present, alcohol treatment effects were isolated as follows. The two control groups never differed significantly from each other on any of the parallel-bar measures (and their means never differed by more than 12%). Consequently, the two control groups were combined (for power and clarity) for the follow-up tests of alcohol-induced behavioral deficits relative to controls, and these comparisons against the combined control mean were made by using Fisher's LSD tests. The cerebellar measures (absolute weight, ratio of cerebellar weight to body weight, total Purkinje cell number) were analyzed with one-way ANOVAs with follow-up group comparisons using Fisher's LSD tests. In addition, the intrasubject coefficient of error (CE) for the optical fractionator counts was calculated according to the method presented by West et al. (37).

RESULTS

Growth

Somatic growth of these rats has been reported in detail in a recent publication addressing other outcomes (16). All four artificially reared groups were delayed in growth relative to suckle controls after postnatal day 11, but the artificially reared groups did not differ among themselves. The body weights on PD 45 (the day of perfusion) also reflected the same pattern (see Table 1). As reported previously, there were significant group effects on PD 45 body weight, $F(4,93) = 4.13$, $p < 0.01$, owing to the significantly heavier body weight of the SC group relative to the artificially reared groups. Importantly, the three alcohol exposed groups did not differ significantly from the GC group in terms of body weight.

Parallel-Bar Performance

Table 2 presents the mean cumulative number of successful traversals over training days, the mean daily composite performance score and mean trial latencies for the parallel-bar training. There were no main or interactive effects of sex on the primary measures of motor performance (successes, steps, or the composite scores). In fact, the only significant sex difference on this task was for mean trial latencies, $F(1,85) = 9.05$, $p < 0.01$, owing to the shorter latencies of females versus those of males. There were no significant main or interactive effects of group for the latency measure. Because the sex differences in latency were consistent and of the same magnitude for all groups and because the primary performance indicators did not differ as a function of sex, data in Table 2 are combined across the two sexes for clarity of presentation.

The cumulative number of successful traversals increased for all groups over days, $F(2,170) = 397.9$, $p < 0.0001$, but there was a significant main effect of group, $F(4,85) = 3.21$, $p < 0.05$, and a group × day interaction, $F(8,170) = 2.28$, $p < 0.05$, owing to the slower acquisition of the parallel-bar task by the alcohol-treated groups. Group differences emerged by the second day of training, by which time each of the three alcohol-treated groups had accumulated significantly fewer traversals than the two control groups ($p < 0.05$ vs. combined control mean). After 3 training days, the PD 7–9 and the PD 4–9 groups completed significantly fewer traversals than did the controls, but the difference between the PD 4–6 group and controls was no longer significant. Comparable outcomes were obtained when considering maximum gap traversed each day, in which the analysis also yielded a significant effect of group, $F(4,85) = 4.70$, $p < 0.01$, day, $F(2,170) = 246.8$, $p < 0.0001$, and a group × day interaction, $F(8,170) = 2.29$, $p < 0.05$. As for the number of successes, the PD 4–6, PD 7–9 and the PD 4–9 groups traversed significantly smaller gaps (4.9, 4.8 and 4.6 cm, respectively, ± 0.25 cm SEM) than did the controls (5.5 and 5.7 cm ± 0.25 cm for SC and GC, respectively).

As shown in the middle columns of Table 2, the composite performance score also indicated that the three alcohol-treated groups were significantly impaired in this motor performance task. Although these scores increased with training in all groups [main effect of day, $F(2,170) = 46.48$, $p < 0.0001$), the scores of the alcohol-exposed groups were lower than the control groups, yielding a significant main effect of group, $F(4,85) = 3.40$, $p < 0.05$. The main effect of alcohol was due primarily to the significantly lower composite scores of all three alcohol groups as compared with controls on the second training day.

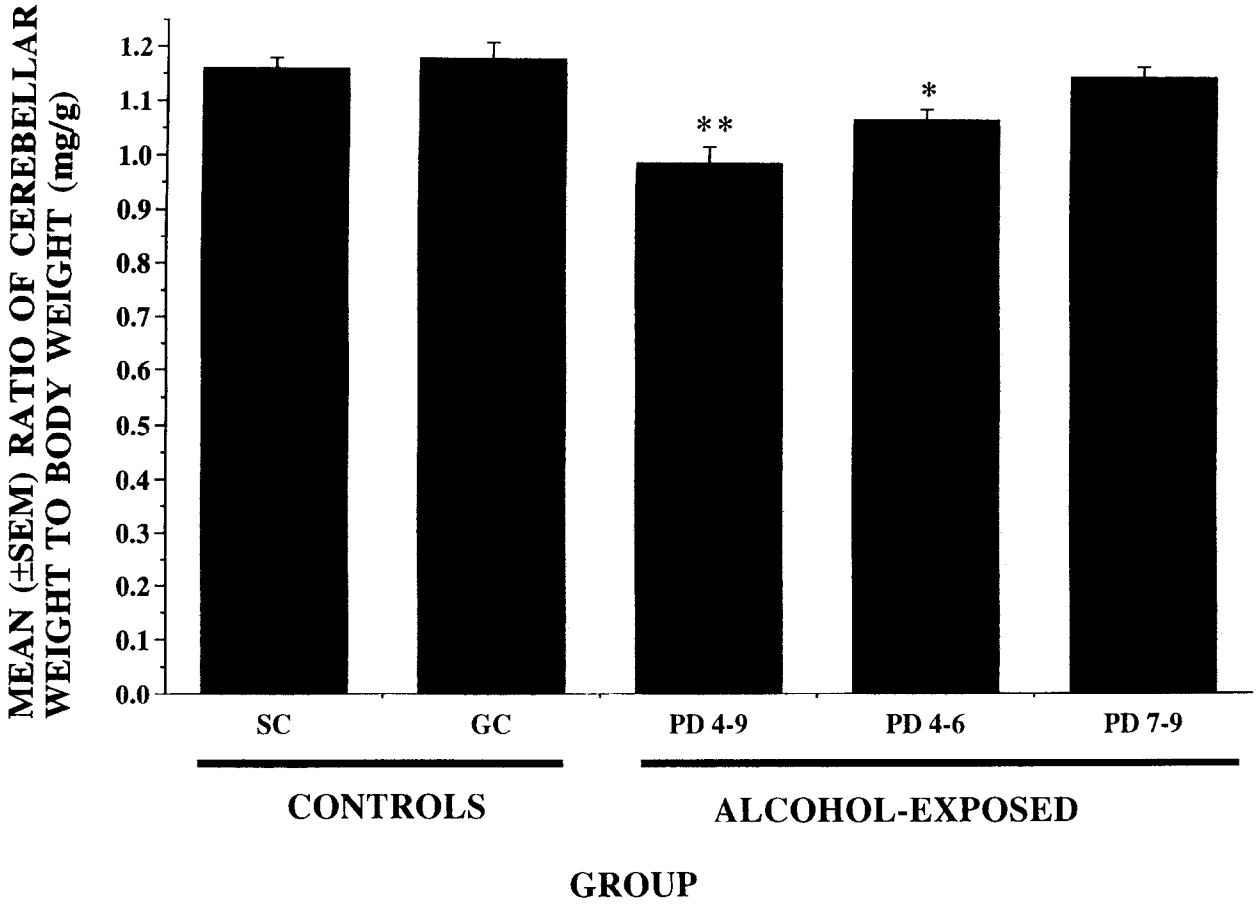

FIG. 1. Mean cerebellar weight:body ratios (±SEM). Data from males and females were combined within group to highlight the significant main effect of alcohol treatment. The PD 4–9 treatment resulted in the most severe restriction of cerebellar growth, and the PD 4–6 (but not the PD 7–9) treatment produced a significant reduction as compared with controls. *Significantly less than the SC, GC and PD 7–9 groups, $p < 0.05$, LSD comparisons. **Significantly less than all other groups, $p < 0.05$, LSD comparisons.

Cerebellar Weights

For the perfused weight of the cerebellum (see Table 1), an ANOVA on absolute weights confirmed significant treatment effects, $F(4,88) = 29.97$, $p < 0.0001$, along with a significant effect of sex, $F(1,88) = 15.51$, $p < 0.0001$; the group × sex interaction was not significant. Regardless of sex, the absolute cerebellar weight of the SC group was significantly heavier than that of the artificially reared groups, in keeping with their heavier body weights at the time of perfusion. However, alcohol treatment on PD 4–9 or PD 4–6 significantly restricted cerebellar weight relative to the GC and the SC groups. The PD 4–9 group had significantly lower cerebellar weights than did all other groups. The PD 4–6 group cerebellar weights were significantly reduced as compared with both control groups, whereas the PD 7–9 group was not different from the GC group. When cerebellar weight was expressed as a ratio to body weight, differences between the SC and GC groups were eliminated, yet significant effects of alcohol treatment effects remained, $F(4,88) = 29.97$, $p < 0.0001$, along with the significant sex effect, $F(1,88) = 15.51$, $p < 0.0001$. The main effect of treatment on these cerebellar weight ratios, shown in Fig. 1 with data collapsed across sex, was the result of significant reductions in the weight ratios of the PD 4–9 and the PD 4–6 groups as compared with controls and with the PD 7–9 group. The cerebellar weight ratio of the PD 4–9 group was significantly lower than that of all groups.

Purkinje Cell Counts

The section sampling fraction of every 14th section yielded a total number of sections between 11 and 13 per cerebellum. The average section thickness per cerebellum after staining was 17.8–20.5 μm (from a nominal cutting thickness of 55 μm) and did not differ among groups. The average total number of cells per cerebellum counted for the five groups ranged from 149 (PD 4–9) to 255 (SC). The sampling strategy yielded an overall average intrasubject CE of 0.06 for total neurons. The mean and range of CEs for neurons for each group were 0.052 for SC (0.035–0.071), 0.065 for GC (0.043–0.097), 0.064 for PD 4–6 (0.040–0.116), 0.042 for PD 7–9 (0.035–0.069) and 0.048 for PD 4–9 (0.047–0.070). CEs of this magnitude are within the range recommended for efficient stereological cell counting because intrasubject error of 5–10% is sufficiently precise to detect treatment effects that are biologically relevant (21,37).

Significant group differences in the total number of Purkinje cells estimated by using the stereological optical fractionator method were confirmed by a 5 (group) × 2 (sex) ANOVA

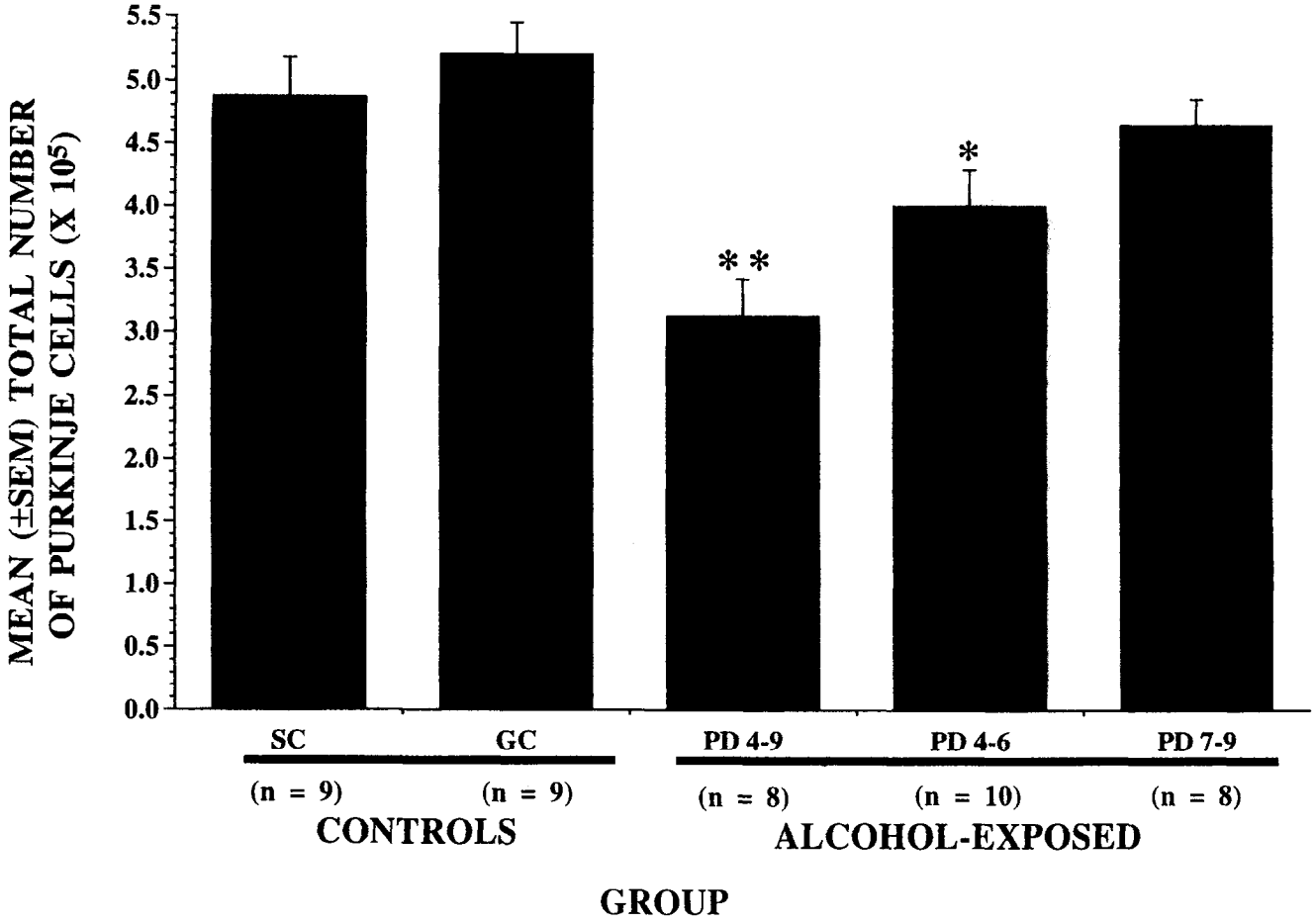

FIG. 2. Total Purkinje cell number (±SEM) determined by using the stereological optical fractionator counting method. Group numbers indicated un the X axis are for males and females combined. The ANOVA yielded a significant group effect without a main or interactive effect of sex. *Significantly less than the SC and GC groups, $p < 0.05$, LSD comparisons. **Significantly less than all other groups, $p < 0.05$, LSD comparisons.

that yielded only a significant main effect of group, $F(4,34) = 8.41$, $p < 0.0001$. Post hoc LSD tests on group means (sexes combined; see Fig. 2) indicated that the PD 4–9 group had significantly ($p < 0.05$) fewer Purkinje cells than did all other groups and that the PD 4–6 group had significantly fewer Purkinje cells than did either control group. The SC, GC and PD 7–9 groups did not differ significantly from each other. Thus, the PD 4–9 exposure and the PD 4–6 exposure resulted in significant Purkinje cell loss as compared with controls (to 68% and 86% of GC, respectively), but the extent of loss was significantly more severe with the 6-day exposure. These are in contrast with the lack of a significant effect of the PD 7–9 alcohol treatment (98% of GC Purkinje cell number).

DISCUSSION

With respect to the original goals of this experiment, three outcomes confirmed the predicted effects. First, the binge treatments over PD 4–9, which did produce mean peak BACs within the range often achieved by human alcohol abusers (ca. 250 mg/dl) produced significant cerebellar damage and motor performance deficits. Second, temporal windows of vulnerability to alcohol induced Purkinje cell loss during this neonatal period were confirmed because exposure on PD 4–6 resulted in significant Purkinje cell depletion, whereas exposure on PD 7–9 did not. Third, the extent of Purkinje cell depletion resulting from exposure on PD 4–9 was significantly greater than either 3-day exposure. With regard to the extent of Purkinje cell loss, the degree of loss produced by the PD 4–9 and the PD 4–6 treatments must be considered as within the pathological range, but they did not reach the severity of Purkinje cell loss induced by the much more intense binge exposure on PD 4/5 of our previous report (33). In that study (in which peak BACs exceeded 350 mg/dl), Purkinje cell numbers were reduced to 55% of the GC group. Nevertheless, the degree of Purkinje cell loss and cerebellar weight restriction produced in the present study establishes a clear risk of cerebellar damage as a consequence of even limited binge exposures during this period of development. The present study also confirms that the extent of such cerebellar damage is a function of the timing of the exposure and the duration (or number) of binge exposures in addition to the peak BACs attained (18).

Two other outcomes were unexpected. The two 3-day alco-

hol treatments produced comparable deficits in parallel-bar motor performance, although only the PD 4–6 binge exposure produced significant Purkinje cell depletion. In addition, the degree of acquisition impairments on the parallel-bar task produced by either 3-day exposure was not significantly different from that produced by the full 6-day exposure. Rather than the expected close association between alcohol-induced motor performance deficits and Purkinje cell loss, this study demonstrates that alcohol-induced motor performance deficits could be detected even in the absence of Purkinje cell depletion in the cerebellum.

This uniform impairment in parallel-bar motor performance, regardless of the timing or duration of neonatal alcohol exposure and regardless of the extent of alcohol-induced Purkinje cell depletion, contrasts with our previous study (34), which found that 2 days of heavy binge exposure on PD 4/5 (and much higher peak BACs) produced significantly more severe parallel-bar deficits than did similar exposure on PD 8/9. The different outcomes between the later binge exposures of the two studies may be a function of the additional day of exposure and/or the initiation of exposure on PD 7 in the PD 7–9 group in the current study, either of which may impart significant risk for motor performance impairments. Perhaps of greater relevance, the treatments of the present study produced BACs more representative of human binge episodes than did our previous study, yet the motor performance deficits were still detectable, regardless of the timing or duration of exposure used.

The obvious implication of the findings of the present study is that the motor deficits measured in this task are not tightly linked to the number of Purkinje cells surviving the alcohol insult. This does not necessarily imply, however, that Purkinje cell depletion is irrelevant to performance of this task. Those rats having clear alcohol-induced depletion of Purkinje cells also fell below the normal range of performance in the parallel-bar task. Rather, the lack of a strong link between Purkinje cell number and motor performance was mainly a consequence of impaired performance of alcohol-exposed animals that had a near-normal complement of Purkinje cells (usually from the PD 7–9 group).

One of the alternative implications of the significant motor performance impairment in the PD 7–9 group is that the treatment affected other structural and functional neural components (either in cerebellum or in other central nervous system regions) that make important contributions to performance on this task but that were not characterized in this study. Motor coordination and execution of learned motor skills certainly involve many interacting neural systems, some of which may be vulnerable during the early neonatal period (e.g., cerebellar cortex) and others that may be vulnerable during the later period. Interestingly, the previously published Morris maze studies with these animals (16) have indicated that for males place learning deficits were significantly more severe following PD 7–9 exposure than following PD 4–6 exposure (females were impaired only with the 6-day exposure). Because the temporal pattern for spatial learning effects did not match that for Purkinje cell loss nor that for parallel-bar performance, the implication is that individual fetal alcohol cases will be a complex composite of neurobehavioral effects in which the specific characteristics will depend in part on the developmental timing and number of episodes of the alcohol exposure.

The parallel-bar task has obvious motivational aspects (associated with balancing at a height) that could have differed as a function of treatment, and differences in elicited fear or stress responses may have differentially affected performance. However, the absence of significant group differences in mean latencies to complete the traversals suggests that any presumed differences in fear or stress did not translate into group differences in speed of initiation and execution of the task. However, although the parallel-bar task is sensitive to the neonatal alcohol insult, it may not be the most appropriate task to examine correlates of alcohol-induced Purkinje cell loss. This task measures a rather easily acquired coordinated movement rather than the stable (asymptotic) performance of a complex motor learning task. As such, it may not be the optimal task to assess correlations with permanent neuronal depletion in the cerebellum. Perhaps more specific motor learning tasks that involve learned performance of complex sequences of ballistic movements (5) or that assess extinction of previously learned motor responses after acquisition has stabilized (27) may be more appropriate to evaluate the role of cerebellar cortical cell loss.

A lesson reiterated from the impaired motor performance of all three alcohol-exposed groups is that behavioral measures of the long-term teratogenic potential of alcohol are an important component of any assessment of fetal alcohol effects. If only Purkinje cell number (or cerebellar growth) were evaluated, the data would indicate that alcohol exposure occurring after the onset of Purkinje cell dendritic development carries less risk than exposure during the initial period of dendritic outgrowth. However, the behavioral measures clearly indicate that adverse outcomes in motor performance are still evident with this later exposure. As is often pointed out (35), behavioral outcomes are often more sensitive measures of teratogenic effects than are morphological outcomes and need to be assessed (in conjunction with central nervous system measures) as indicators of teratogenic consequences sui generis and as well as being used as potential functional correlates of neurobiological outcomes. For fetal alcohol effects, adverse behavioral outcomes constitute some of the most pertinent data that bear on teratogenic potential, particularly when the behavior being considered has obvious links to known human behavioral dysfunction (as is the case with impaired motor performance and coordination).

Given that neuronal loss is likely one of the more severe consequences to the nervous system, these data confirm that the timing of exposure to alcohol can be a crucial determinant of the potential for damage to the developing brain. In the rat, bingelike exposure causes more severe Purkinje cell depletion when administered during neonatal periods of differentiation than when administered during prenatal periods of neurogenesis (25). The present and previous (12,22,33) studies confirm that the first postnatal week in rats is a period of enhanced vulnerability to alcohol-induced after mitotic Purkinje cell loss, a time when dendritic outgrowth is rapidly progressing. Identifying the brain developmental events that are responsible for this time-limited vulnerability will constitute a crucial step toward understanding the mechanisms of and variation in outcomes of this particular human fetal alcohol effect. One specific prediction that emerges from this study is that heavy binge drinking episodes that occur during the comparable peak initial period of human Purkinje cell dendritic outgrowth, which occurs roughly between fetal weeks 25 and 32 (38), are likely to be associated with the kind of severe cerebellar structural deficits observed in recent magnetic resonance imaging studies of FAS children (26,32).

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