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Chlordiazepoxide-Induced Spatial Learning Deficits: Dose-Dependent Differences Following Prenatal Malnutrition

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TONKISS, J., P. L. SHULTZ, J. S. SHUMSKY, T. A. FIACCO, M. VINCITORE, D. L. ROSENE AND J. R. GALLER. *Chlordiazepoxide-induced spatial learning deficits: Dose-dependent differences following prenatal malnutrition.* PHARMACOL BIOCHEM BEHAV **65**(1) 105–116, 2000.—The sensitivity of prenatally protein-malnourished rats to the amnestic properties of the benzodiazepine (BZ) receptor agonist, chlordiazepoxide (CDP), was studied in the male offspring of rats provided with a protein-deficient diet (6% casein) for 5 weeks prior to mating and throughout pregnancy. Rats were tested during acquisition of the submerged platform version of the Morris water maze task using three systemic doses of CDP (3.2, 5.6, and 7.5 mg/kg IP) at two ages (day 30 and day 90). At 30 days, prenatally malnourished rats showed less sensitivity to the amnestic effect of the 5.6-mg/kg dose when compared with well-nourished controls by displaying shorter swim paths during acquisition and a more selective search of the target quadrant upon removal of the platform (probe trial). At 90 days, prenatally malnourished rats again showed less sensitivity to CDP at a dose of 5.6 mg/kg, but more sensitivity to the 3.2-mg/kg dose (indicated on the probe trial). No obvious relationship was identified between the nutritional group differences in behavioral sensitivity to CDP at 90 days and their BZ receptor density in the hippocampus or medial septum. It can be concluded that prenatal malnutrition alters the amnestic response to CDP in a dose-dependent and developmentally specific manner, thus providing further support for functional changes within the GABAergic system subsequent to malnutrition.

Prenatal protein malnutrition Protein restriction Pregnancy Benzodiazepine receptor agonist CDP Morris maze Water maze Rats

A number of previous studies [reviewed in (6)] have examined behavioral sensitivity to centrally acting drugs in rodents that have experienced malnutrition during the early phases of their development (i.e., prenatal and early postnatal life). Although drugs that exert their actions in the catecholaminergic, GABAergic, serotonergic, opioid, cholinergic, and glutamatergic systems have been investigated, those drugs that act at the $GABA_A$ receptor complex have been studied most extensively (2–5,8,10–13,15–17,22,24,27,36). Benzodiazepine (BZ) receptor agonists (e.g., diazepam and chlordiazepoxide (CDP)) are among those compounds that facilitate GABA-mediated inhibition upon binding to the BZ recognition site on the $GABA_A$ receptor complex. Behaviorally, these agents are known to possess anxiolytic, amnestic, and sedative properties. Despite a diversity of methods, including the malnutrition procedure (i.e., protein–calorie restriction, large litter rearing, or protein malnutrition), the timing of malnutrition, and the model of anxiety employed, the finding that malnutrition gives rise to a reduced sensitivity to the anxiolytic effects of BZ receptor agonists has proven reliable and consistent

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[reviewed in (6)]. However, studies of the effects of BZ receptor agonists on learning and memory in malnourished animals have been both few in number and variable in their outcome (2,5,13,24). Moreover, these studies used only a single dose of CDP, administered to adult rats, and all but one (13) employed highly aversive footshock procedures to which malnourished animals are known to be hyperresponsive (26,35,37). Thus, the degree of sensitivity of previously malnourished animals to the amnestic properties of BZs remains an open question. The behavioral response of malnourished animals to one aspect of a drug may be very different to their response to a different property of the same compound. Thus, although the sensitivity of malnourished animals to BZ treatment has been well-characterized in anxiety tests, a systematic analysis of the BZ dose–response relationship in learning paradigms is required to test whether the findings generalize to a different attribute of this drug class.

The Morris water maze (30,31) is commonly used as a test of cognitive functioning in rodents. It has proven sensitive in revealing impaired performance following the administration of centrally acting drugs and following damage to the septohippocampal system (9). Animals are placed into a large tank of water where they swim to find a "hidden" escape platform. The platform is located just below the surface of the water, at a fixed position relative to remote visual cues in the vicinity of the tank. The flexible use of multiple cues is required for the animals to navigate to the platform because different start positions are employed. This test has several advantages for drug work, especially in malnourished animals. First, it is only mildly aversive compared to footshock. Second, there is no requirement for food or water deprivation, which could differentially alter drug distribution, metabolism, or elimination. Third, the animals are capable of effective swimming and remain motivated to locate the escape platform, even at doses of drugs that cause significant motoric effects. Finally, at ages ranging from postnatal day 21 to 220, prenatally malnourished rats do not differ from well-nourished controls in the acquisition of this task (40), except under unusual prenatal conditions (41), and thus, the two nutritional groups begin from the same baseline levels of performance.

Numerous regions of the brain exhibit a high density of BZ receptors, and several of these are thought to subserve mnemonic functions. To identify the neuroanatomical specificity underlying the amnestic response to intracranial infusions of a BZ receptor agonist, a recent study (28) examined six BZ receptor-rich sites (frontal cortex, nucleus basalis magnocelluaris/substantia innominata, amygdala, medial septum, hippocampus, and cerebellum). Only direct infusion of CDP into the medial septum generated impairment in the acquisition of the Morris water maze task, leading the authors to suggest that the medial septum was a critical structure for the amnestic action of CDP. Although infusion of CDP into the hippocampus was without effect, the authors drew attention to the fact that the small injection volume used was unlikely to have reached the entire hippocampus, leaving the role of the hippocampus in CDP-induced amnesia unresolved.

Here we report the effect of CDP, administered systemically at a range of doses (3.2, 5.6, and 7.5 mg/kg) and examined at two ages (postnatal days 30 and 90), on the acquisition of the Morris water maze task in prenatally malnourished and well-nourished (control) male rats. To assess the possibility that altered sensitivity to the amnestic properties of CDP following prenatal malnutrition was related to changes in BZ receptor densities in critical areas of the brain, BZ receptor binding in the medial septum and in the hippocampus was quantified in representative subsets of animals behaviorally characterized at postnatal day 90.

METHOD

Housing Conditions

The animal quarters were maintained at a temperature of 73°F (±3) and at 45–55% humidity. A reverse 12 h night (0800–2000)/12 h day (2000–0800) light cycle accommodated behavioral observations to the active waking period of the rat. Red fluorescent lighting during the dark portion of the cycle provided continuous dim illumination.

Nutritional Treatment

Five weeks prior to mating, nulliparous female rats (Sprague–Dawley VAF plus; Charles River Laboratories, Kingston, MA) were obtained and allowed ad lib access to one of two isocaloric diets (Teklad, Madison, WI). The diets were formulated to be of adequate protein (25% casein) or low protein (6% casein) content [detailed description given in (19)]. Males obtained from the same source were acclimated to the experimental diets of the females for 1 week prior to mating. Each male was mated with two females receiving the same dietary treatment, over a period of 10 days. The presence of sperm in a vaginal smear determined whether mating had occurred. One week prior to the projected delivery date the females were individually caged in polycarbonate breeding cages (51 × 41 × 21 cm; Lab Products Inc., Maywood, NJ). Following parturition, litters were culled to eight pups (six males and two females) and fostered to well-nourished mothers who had given birth no more than 24 h previously. Pups born to dams provided with the 6% casein diet and crossfostered to lactating dams given the 25% casein diet were assigned the abbreviation "6/25" (prenatally malnourished). Pups born to dams provided with the 25% casein diet and fostered to other lactating dams fed the 25% casein diet were assigned the abbreviation "25/25" (controls). A more detailed description of the nutritional, mating, and fostering procedures is given in a previous paper (39). All rats were weighed at birth and on the day of behavioral testing, using an electronic balance (Ohaus, GT 4000). After weaning at 21 days all offspring were pair-housed and given ad lib access to Purina rat chow (Formula 5001). The animals continued to be pair-housed throughout behavioral testing.

Spatial Navigation Apparatus and General Procedure

A 1.5-m diameter, 45-cm deep, circular water maze (30,31) was used, together with a computer tracking system (Polytrack software program, San Diego Instruments), which monitored and recorded the swim path of the rats for later analysis. The maze was constructed of white plastic and filled with water to a depth of 26.5 cm. The water temperature was adjusted to 25°C (±0.5). A circular platform, 25 cm high and 12 cm in diameter, was placed into the tank at a fixed location in the center of one of four imaginary quadrants. The water was rendered opaque with the addition of approximately 1.5 liters of milk. As the platform could not be seen or detected by smell, the rats were required to learn to navigate to the platform by using remote (distal) cues (e.g., posters, cabinets, sinks, etc.) in the vicinity of the tank (4–8 feet). Rats were first given 1 min upon the platform before undergoing three swim-training trials, during which the rat swam to the platform with assistance from the experimenter. After the third training trial, the rat was taken from the platform and released into

the water at one of three start positions, facing the maze wall. The start positions were all approximately equidistant from the platform, located immediately adjacent to the wall in the center of the three quadrants that did not contain the platform. The rat was allowed to swim for up to 1 min in an attempt to locate the platform and thus escape. If it failed to locate the platform within that time, the experimenter assisted escape. Six test trials (two trials from each of the three start positions, presented in a random order) were given on each of 4 consecutive test days. An intertrial interval of 35 s was imposed while the rat was on the platform. Escape latency and distance traveled were measured on each trial. On the 25th trial (i.e., the seventh trial of the fourth day) the platform was removed and the rat was released into the quadrant diagonally opposite to the quadrant that had previously contained the platform. The rat was allowed to search for the platform for a period of 1 min ("probe" trial). The distance traveled and the time spent searching during the probe trial, in each of four quadrants, was recorded on this trial, and each measure served as an index of search selectivity. Annulus crossings (i.e., crossings of the former platform location and of corresponding areas in the other three quadrants) were also recorded. The rats were coded such that the researcher placing the rats into the pool was blind to the experimental condition of the subject.

CDP Dose–Response Determination During Acquisition of a Spatial Task at Two Ages: Day 30 (Experiment 1) and Day 90 (Experiment 2)

Subjects and drug administration. The effect of CDP on spatial learning in the Morris water maze was examined at two ages: at day 30 (juvenile) in Experiment 1, and at day 90 (adult) in Experiment 2. The studies were performed separately to permit a within-litter design across the different drug dosages, at each age. Thus, for each age, 10 6/25 and 10 25/25 litters each contributed one male subject for testing at one of four doses of CDP HCl (Sigma Chemicals, St. Louis, MO): 0 (saline), 3.2, 5.6, and 7.5 mg/kg. The drug was dissolved in 0.9% saline, and administered in a volume of 1 ml/kg by intraperitoneal injection (IP), 20 min prior to testing in the Morris water maze. This time period also allowed the rats to adapt to white light before behavioral testing commenced. Trials were administered according to the protocol given above, with the platform located in the SE quadrant. One rat from the 25/25 saline group was eliminated from Experiment 1 due to accidental loss of data.

Receptor binding. The density of BZ receptors within the hippocampal formation had previously been shown to be greater at postnatal day 15 following prenatal malnutrition (7). The hippocampal formation and the medial septum are brain regions thought to be critically involved in the amnestic response to BZs (28,38). We determined whether there was any relationship between the behavioral response to BZs and the density of BZ receptors within these regions, in the 90-day-old subjects tested in Experiment 2, by selecting two rats per nutritional group per dose as exhibiting the most representative behavioral response for their group (based upon probe trial data). After at least 3 weeks following their last drug exposure, they were deeply anesthetized (45 mg/kg, sodium pentobarbital) and perfused intracardially with cold Krebs–Henseleit buffer (4°C). The brain was rapidly removed, blocked (i.e., brainstem and cerebellum were removed), flash frozen by slow immersion into −60°C isopentane, and stored at −80°C until sectioning. A Hacker/Brights motorized cryostat was used to cut 15-μm horizontal sections, which were thaw mounted onto gelatin or poly-L-lysine subbed slides and stored, frozen, at −20°C, until processed. Two adjacent series, of adjacent sections, through the entire hippocampal formation (which included all of the medial septum) from all of the rats were assayed together—one series for total binding and the other for nonspecific binding. For receptor-binding assays, the sections were rapidly thawed to prevent the tissue from accumulating moisture. Total [^{3}H]flunitrazepam ([^{3}H]FLU, specific activity, SA = 84.5 Ci/mmol; New England Nuclear) binding (i.e., specific and nonspecific) was achieved by incubating the sections in 2 nM [^{3}H]FLU in 0.17 M Tris-HCl buffer (pH 7.4) for 40 min at 0°C. A measure of nonspecific binding was obtained by adding a competitive receptor blocker (Clonazepam), at high concentration (1 μM—sufficient to displace 95% of the tritiated ligand from the specific binding sites), to the assay for an adjacent series of sections. For both assays, sections were agitated constantly at low speed on a shaker table throughout incubation. After incubation, the sections were rinsed twice in 50 mM Tris-HCl buffer solution (1 min at 0°C) and once in distilled water (10 sec at 0°C). The slides were rapidly dried under a stream of cool air (4–5 min), stored overnight in a desiccator, and then loaded into X-ray cassettes with a set of tritium microscales (20). The slides were then apposed to tritium sensitive film (Amersham) and allowed to expose at room temperature in the dark for 2 weeks. Adjacent "nonspecific" sections were always placed next to the corresponding "total" sections on the film. The films were developed using Kodak D19. Total and nonspecific images of the sections, together with the tritium standard on each film, were digitized using "Inquire" software (Loats Associates, Westminster, MD) for quantitative analysis. The tritium standards on each film were sampled to construct a calibration table to which all images on the same sheet of film were "tagged." Upon sampling, the calibration table automatically converted optical density into fmol/mg tissue, based upon the manufacturer's values for the standards. The hippocampus and medial septum were sampled in all total binding images by this method. Due to difficulty in establishing the exact boundary of the medial septum, triplicate samples were taken from each section and averaged together. Nonspecific images were sampled and subtracted from total binding only if visible images appeared on the film. For each animal, specific fmol values from each quantified section were averaged together, and analysis was performed upon these values to determine the presence of any group differences.

Independent Replication of the (a) 3.2 mg/kg and (b) 5.6 mg/kg CDP Dose for Day 90 Rats (Experiment 3)

In Experiment 2, a differential dose effect between the two nutritional groups was only obtained for one measure (annulus crossings during the probe trial). Because the 6/25 rats proved to be less sensitive than the 25/25 rats to the 5.6-mg/kg dose of CDP, and perhaps more sensitive to the 3.2-mg/kg dose of CDP, independent replications were deemed necessary to confirm these observations. Two naive sets of well-nourished and prenatally malnourished rats were derived according to the nutritional treatment protocol given above. The rats were then tested in adulthood according to a slightly modified behavioral protocol. Rather than 4 consecutive days of testing (six trials per day), the rats were given 2 consecutive daily days of testing with 12 trials per day (maximum of 60 s per trial), followed by a 60-s probe trial (i.e., 13th trial of the

2nd day). This was done to reduce the number of CDP injections, thus lessening the chance of significant receptor changes due to repeated drug exposure, while providing the same absolute number of test trials. All other parameters remained the same as before. Within each replication, 10 6/25 and 10 25/25 litters each provided two male subjects for testing. One male was assigned to receive saline injections and the other to receive CDP at a dose of 3.2 mg/kg [replication (a)], or 5.6 mg/kg [replication (b)]. Thus, a within-litter design was employed as before. The rats in replication (a) were 100 days of age at the start of testing and the rats in replication (b) were 90 days of age at the start of testing. In replication (b), one 25/25 and one 6/25 CDP rat were eliminated due to administration of incorrect injections.

Statistics

The body weights of prenatally malnourished and well-nourished "control" rats were compared at birth (day 0) and at testing (day 30 and 90) using one-way ANOVA's. Separate ANOVAs were applied to the three different age groups due to dissimilar variances. The data for measures of escape latency, distance traveled, and swim speed were first converted to three-trial averages (blocks) within each day of testing. The data were then subjected to four-way ANOVA's (nutritional group × dose × day × block). Nutritional group and dose were between-subject variables and day (four levels) and block (two levels) were within-subject variables. Because distance traveled and escape latency were highly correlated, the same pattern of results was indicated for both measures. Hence, only distance traveled will be reported below. On the probe trial, the distance traveled within the target (SE) quadrant (as a percentage of the total distance traveled in all four quadrants: SE, SW, NE, and NW) was compared across treatment groups using two-way ANOVAs [nutritional group × dose (between-subject variables)]. Within each group, single sample *t*-tests were also applied to determine whether the percentage of distance traveled in the (SE) target quadrant was significantly greater than that expected by chance (i.e., 25%). In Experiment 2 (adults), annulus crossings were compared across treatment groups using three-way ANOVAs [quadrant (within-subject) × nutritional group (between-subject) × dose (between-subject)] and in Experiment 3, these data were analyzed by two-way ANOVAs [quadrant (within-subject) × nutritional group (between-subject)] for each replicated dose. Within each group, the number of annulus crossings was also compared between the four quadrants using univariate ANOVAs. This was done to determine the specificity of the subjects' searching pattern. No analysis of annulus crossings was performed in Experiment 1 due to the presence of an unusually large number of zeros (especially at the two highest CDP doses). Where main effects or interactions attained significance, protected two-tailed *t*-tests, based on the error terms and degrees of freedom from the ANOVA, were used to make subsequent comparisons among the means of interest. These comparisons were protected from large type I errors by the requirement that the preliminary *F*-test meet the α criterion (14,25). Greenhouse–Geisser adjusted *p*-values are reported in those repeated-measures analyses where sphericity violations were indicated. Total [^{3}H]FLU binding within the hippocampal formation and medial septum was analyzed by separate two-way ANOVAs [nutritional group × dose (between-subject variables)].

RESULTS

Experiment 1—Juvenile Rats (Day 30)

Body weight. The body weight of the rats at birth (day 0) and at day 30 is given in Table 1. ANOVA indicated that, at day 0, the body weight of the 6/25 rats was significantly less than that of the 25/25 rats, $F(1, 18) = 17.01$, $p < 0.001$. Although the body weight deficit decreased from 16% at day 0 to 13% at day 30, the 6/25 rats continued to weigh significantly less than the well-nourished controls, $F(1, 18) = 18.35$, $p < 0.001$.

Spatial navigation. The distance traveled during acquisition of the spatial task is given in Fig. 1 (upper panels). Significant reductions in distance traveled were seen across day,

TABLE 1
MEAN BODY WEIGHTS (± SEM)

	Prenatally Malnourished 6/25 (*n*)	Adequately Nourished 25/25 (*n*)	Deficit
Experiment 1 (juveniles)			
Birth (day 0)[1]	5.37 ± 0.20 (10)*	6.39 ± 0.14 (10)	16%
Testing (day 30)[2]	113.25 ± 3.56 (10)*	129.93 ± 1.59 (10)	13%
Experiment 2 (adults)			
Birth (day 0)[1]	5.16 ± 0.18 (10)‡	5.73 ± 0.12 (10)	10%
Testing (day 90)[2]	497.80 ± 7.33 (10)‡	530.38 ± 11.68 (10)	6%
Experiment 3a (adults)[3]			
Birth (day 0)[1]	5.03 ± 0.10 (10)**	6.05 ± 0.18 (10)	17%
Testing (day 100)[2]	525.75 ± 8.33 (10)†	573.65 ± 8.98 (10)	8%
Experiment 3b (adults)[4]			
Birth (day 0)[1]	5.75 ± 0.20 (10)*	6.30 ± 0.17 (10)	9%
Testing (day 90)[2]	468.00 ± 10.94 (10)	474.40 ± 8.30 (10)	1%

*$p < 0.001$, †$p < 0.01$, ‡$p < 0.05$ compared with the 25/25 group (ANOVA).
[1]Litter mean weight for all male pups.
[2]Litter mean weight for the males subjected to behavioral testing.
[3]Replication of the 3.2 mg/kg CDP dose.
[4]Replication of the 5.6 mg/kg CDP dose.

$F(3, 213) = 101.85$, $p < 0.001$, and blocks, $F(1, 71) = 87.56$, $p < 0.001$, and dose-dependent impairments were observed with CDP, $F(3, 71) = 43.71$, $p < 0.001$. A significant effect of nutritional group, $F(1, 71) = 9.29$, $p < 0.01$, indicated that the 25/25 rats swam farther (mean = 1207 cm) than the 6/25 rats (mean = 1017 cm). However, the presence of a significant nutritional group × dose interaction, $F(3, 71) = 2.83$, $p < 0.05$, indicated that this effect was primarily due to the 25/25 rats swimming significantly farther (mean = 1502 cm) than the 6/25 rats (mean = 1134 cm) under the 5.6 mg/kg dose of CDP ($p < 0.001$). There were no significant differences between the two nutritional groups under the saline condition, or the 3.2 or 7.5 mg/kg doses of CDP. Significant day × dose, $F(9, 213) = 6.91$, $p < 0.001$, and day × block × dose, $F(9, 213) = 2.43$, $p < 0.02$, interactions were also indicated.

BZs may impair motor performance, especially at high doses. To assess possible differences in motor performance between 6/25 and 25/25 rats after administration of CDP, swim speed was analyzed. Swim speed, illustrated in the lower panels of Fig. 1, showed the pattern of findings expected to be associated with dose-related impairment of the rats' ability to find the platform. The more impaired the rat, the longer was their swim path, leading to a greater level of fatigue and a greater decline in swim speed. This observation was confirmed by an ANOVA, which showed that swim speed decreased over days, $F(3, 213) = 23.45$, $p < 0.01$, and across blocks, $F(1, 71) = 37.00$, $p < 0.01$, with the decrease across blocks declining between day 1 (i.e., blocks 1 and 2) and day 4 (i.e., blocks 7 and 8), as confirmed by a significant day × block interaction, $F(3, 213) = 7.40$, $p < 0.001$. The decline across blocks was pronounced in the high dose group (Δ block 1-block 2 = 2.41 cm/s) and not different in the saline group (Δ block 1-block 2 = −0.34 cm/s), which emerged as a significant block × dose interaction, $F(3, 71) = 8.35$, $p < 0.001$. There was a significant effect of nutritional group, $F(1, 71) = 23.57$, $p < 0.01$, due to the 25/25 rats swimming significantly faster (mean = 28.59 cm/s) than the 6/25 rats (mean = 26.65 cm/s), and a significant block × nutritional group interaction, $F(1, 71) = 4.23$, $p < 0.05$), due to a greater decline in swim speed over blocks in the 25/25 group (Δ = 1.65 cm/s) compared with the 6/25 group (Δ = 0.79 cm/s). Importantly, there were no significant nutritional group × dose or nutritional group × dose × block interactions, indicating that any dose-induced motor impairments that might have been present were similar across the two nutritional groups.

The distance traveled per quadrant on the probe trial is shown in Fig. 2. Comparison of the distance traveled in the (SE) target quadrant (percentage of total distance traveled in all four quadrants) revealed a significant effect of drug dose, $F(3, 71) = 8.01$, $p < 0.01$, and a significant dose × nutritional group interaction, $F(3, 71) = 3.28$, $p < 0.05$. Post hoc analyses revealed that the 6/25 rats showed a significantly greater amount of searching in the target quadrant than the 25/25 rats at a CDP dose of 5.6 mg/kg ($p < 0.02$). There were no differences between the two Nutritional Groups under the saline condition or the 3.2 or 7.5-mg/kg Doses of CDP. All 6/25 groups, except for the 7.5-mg/kg CDP dose group, and all 25/25 groups, except for the 5.6-mg/kg CDP Dose group, traveled a greater percentage of distance in the target quadrant than that expected by chance.

In summary, at 30 days of age, the 25/25 rats showed a greater impairment of place learning than the 6/25 rats under a 5.6-mg/kg dose of CDP. This impairment manifested itself as a greater distance traveled during acquisition of the task and as less searching in the target quadrant on the probe trial.

Experiment 2—Adult Rats (Day 90)

Body weight. The body weight of the rats at day 0 and at day 90 is given in Table 1. At day 0, the body weight of the 6/25 rats was significantly less than that for the 25/25 rats, $F(1, 18) = 7.16$, $p < 0.02$. Although the body weight deficit decreased from 10% at day 0 to 6% at day 90, the 6/25 rats continued to weigh significantly less than the well-nourished controls, $F(1, 18) = 5.59$, $p < 0.05$.

Spatial navigation. The distance traveled during acquisition of the spatial task is given in Fig. 3 (upper panels). Unlike the findings obtained at day 30, no significant difference in dose response was seen between the 6/25 and 25/25 rats. There was, however, a significant effect of drug dose, $F(3, 72) = 18.21$, $p < 0.001$, due to the rats swimming greater distances (i.e., distance traveled) with increasing doses of CDP (mean distance: saline = 398 cm, 3.2 mg/kg = 631 cm, 5.6 mg/kg = 725 cm, and 7.5 mg/kg = 901 cm). The amount of improvement across days was also dependent upon the drug dose, as indicated by a significant day × dose interaction, $F(9, 216) = 3.19$, $p < 0.01$. There were significant effects of day, $F(3,216) = 140.86$, $p < 0.001$, block, $F(1,72) = 74.35$, $p < 0.001$, and a day × block interaction, $F(3,216) = 15.32$, $p < 0.01$, reflecting significant improvements in performance both within and across days.

Figure 3 (lower panels) illustrates the swim speed of the rats during acquisition of the task. Similar to the pattern of results at day 30, there were no significant interactions involving nutritional group and dose, indicating that any motor impairments caused by CDP were similar across the two nutritional groups. Swim speed declined over day, $F(3, 216) = 53.87$, $p < 0.001$, and across blocks, $F(1, 72) = 26.67$, $p < 0.01$, with the decline across blocks lessening between day 1 (i.e., blocks 1 and 2) and day 4 (i.e., Blocks 7 and 8), as indicated by a significant day × block interaction, $F(3, 216) = 9.14$, $p < 0.001$. There was also a significant day × dose interaction, $F(3, 216) = 2.07$, $p < 0.05$. No nutritional group effect was observed in swim speed at day 90.

Figure 4A illustrates the distance traveled per quadrant (percentage of total distance traveled) during the probe trial. All groups showed a selective search of the (SE) target quadrant relative to the other quadrants (with the exception of the 25/25 7.5 mg/kg CDP group, whose data had high variance). This finding indicated that the subjects had acquired a place response despite the obvious impediment to task acquisition afforded by the drug. Analysis of the percentage of distance traveled in the (SE) target quadrant across the treatment groups indicated a significant effect of dose, $F(3, 72) = 3.86$, $p < 0.02$, attributable to a dose-dependent decrease in the amount of searching in that quadrant (mean distance traveled in target quadrant: Sal = 46.4%, 3.2 mg/kg = 41.8%, 5.6 mg/kg = 37.9%, 7.5 mg/kg = 35.0%).

There was no significant effect of nutritional group, and no nutritional group × dose interaction. Analysis of the annulus crossings, however, revealed a different pattern of results (Fig. 4B). The search specificity was first examined within each group by comparing the number of annulus crossings in the target quadrant and of the annuli in the other three quadrants. With the exception of four groups—6/25: 3.2 mg/kg and 7.5 mg/kg CDP, and 25/25: 5.6 and 7.5 mg/kg CDP—all remaining groups showed a significant difference in the amount of annulus crossings in the four quadrants. This difference was due to a significantly greater number of annulus crossings in the target quadrant than in any of the other quadrants ($p < 0.01$). Hence, the 5.6-mg/kg and 7.5-mg/kg dose of CDP elimi-

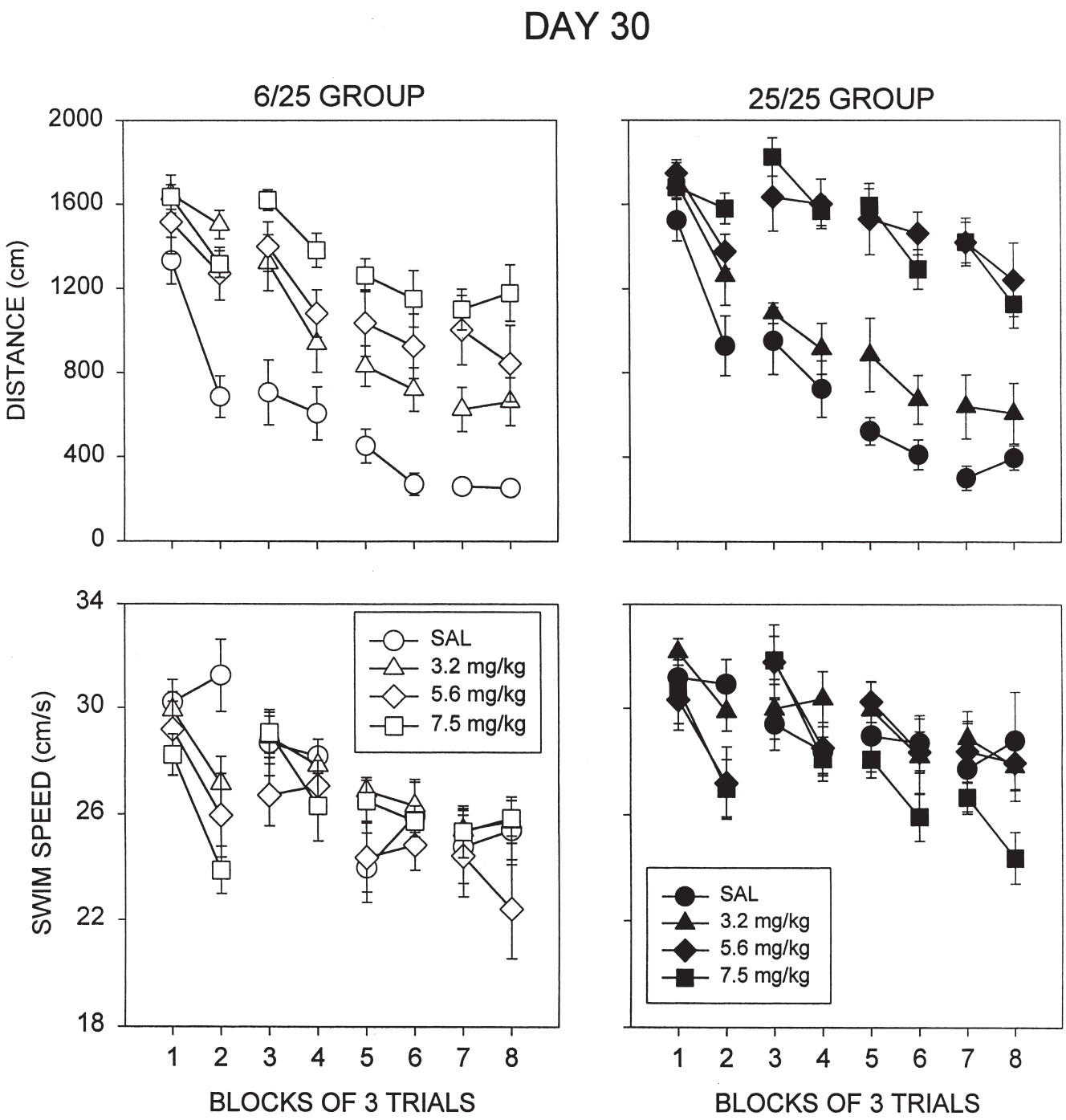

FIG. 1. Experiment 1: effect of CDP on acquisition of the Morris maze in juvenile 6/25 (left panels) and 25/25 (right panels) rats. Upper panels indicate the distance swum to the platform. Lower panels indicate the swim speed.

nated the selective search pattern of 25/25 rats, whereas the selective search pattern of 6/25 rats was disrupted at CDP doses of 3.2 and 7.5 mg/kg. These findings were partially confirmed by comparing the number of annulus crossings in the target quadrant between the 6/25 and 25/25 rats, across the various drug doses. A significant nutritional group × dose interaction, $F(3, 72) = 3.95$, $p < 0.02$, was indicated, due to the 6/25 rats showing significantly greater numbers of annulus crossings in the target quadrant than the 25/25 rats at a dose of 5.6 mg/kg CDP ($p < 0.05$), and tending to show lower numbers of annulus crossings in the target quadrant at a dose of 3.2 mg/kg ($p < 0.1$).

Receptor binding. Figure 5 illustrates that the density of [^{3}H]FLU binding sites within the hippocampus and medial septum was similar across nutritional groups and across CDP dose groups. In the case of the hippocampus, finer grained

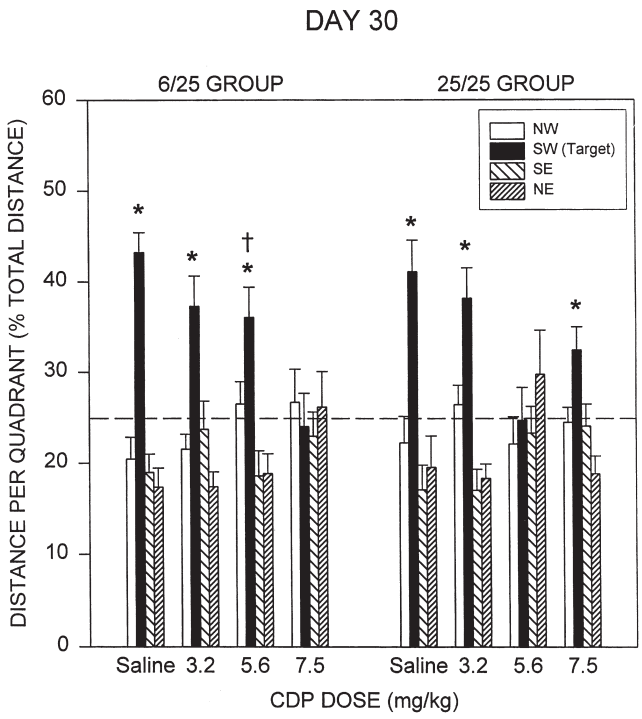

FIG. 2. Experiment 1: effect of CDP on the relative distance traveled per quadrant in juvenile 6/25 and 25/25 rats during the probe trial (platform removed). *Target quadrant greater than chance level (indicated by dashed line), $p < 0.05$. †Target quadrant for the 6/25 group greater than that of the 25/25 group for the same dose of CDP ($p < 0.05$).

analysis of 11 subfields and laminae (data not shown) also failed to reveal any significant differences due to prenatal malnutrition.

Experiment 3—Replication of Critical Doses in Adult Rats (Days 90–100)

Body weight. The body weight of the rats at day 0 and at testing is given in Table 1. The body weight of the 6/25 rats at day 0 was significantly less than the 25/25 rats in both cohorts [replication (a): $F(1, 18) = 23.25$, $p < 0.001$; replication (b): $F(1, 18) = 4.65$, $p < 0.05$. However, by the time of testing at days 90–100, the 6/25 rats no longer differed from the 25/25 rats in replication (b), but continued to weigh significantly less, $F(1, 18) = 15.28$, $p < 0.01$, than the 25/25 rats in replication (a).

Spatial navigation. (a) 3.2-mg/kg CDP dose: as in Experiment 2, there was no significant difference in dose response between the 6/25 prenatally malnourished and the 25/25 well-nourished rats in the distance traveled during acquisition of the task (data not shown). Nonetheless, there was a significant dose effect, $F(1, 35) = 19.33$, $p < 0.001$, due to the drugged animals (mean = 777 cm) swimming significantly farther (i.e., distance traveled) than the saline-injected rats (mean = 475 cm). Performance improved over the two test days, $F(1,35) = 67.04$, $p < 0.001$, and over blocks, $F(3,105) = 65.14$, $p < 0.001$, with smaller improvements across blocks on the second day (mean $\Delta = 232$ cm) when compared with the first day (mean $\Delta = 669$ cm), indicated by a significant day × block interaction, $F(3, 105) = 10.64$, $p < 0.001$. A significant day × block × dose interaction, $F(3,105) = 3.49$, $p < 0.05$, denoted much smaller improvements over blocks in the drugged animals, especially on day 1. With respect to swim speed, a significant effect of nutritional group was observed, $F(1,35) = 6.96$, $p < 0.02$. This finding was due to the 6/25 rats (mean = 28.16 cm/s) swimming significantly faster than the 25/25 rats (mean = 25.42 cm/s). Swim speed declined over blocks, $F(3,105) = 10.76$, $p < 0.001$, with the decline being more pronounced in drugged rats, as indicated by a significant block × dose interaction, $F(3,105) = 4.75$, $p < 0.01$.

Consistent with the probe trail data of Experiment 2, the two nutritional groups did not differ in their dose response with respect to the distance traveled in the target quadrant (percentage of total distance traveled). The only significant effect was one of quadrant, $F(3, 105) = 36.55$, $p < 0.001$, due to a significantly greater distance traveled in the target quadrant than in any of the other three quadrants ($p < 0.01$). However, verification of a differential dose response in the 6/25 and 25/25 nutritional groups emerged on the specificity of search pattern, as indicated by analysis of annulus crossings (Fig. 6A). With the exception of the 3.2-mg/kg 6/25 nutritional group, all groups showed a significant difference in the number of annulus crossings between the four quadrants. In all of these cases, this finding was due to a significantly greater number of annulus crossings in the target quadrant relative to any in the other three quadrants ($p < 0.02$). Hence, these data suggest that selective and localized search patterns were evident in the 25/25 group, but were practically eliminated in the 6/25 group, at a dose of 3.2 mg/kg CDP. It should be noted, however, that specific ANOVA comparison of the number of annulus crossings in the target quadrant across the four treatment groups (i.e., 6/25 saline, 6/25 CDP, 25/25 saline, and 25/25 CDP) failed to reveal any significant differences.

(b) 5.6-mg/kg CDP dose: no significant differences were detected in the distance traveled during acquisition between the prenatally malnourished and well-nourished nutritional groups when administered CDP (data not shown). However, a significant dose effect, $F(1,34) = 94.31$, $p < 0.001$, was evident, due to the drugged animals swimming significantly farther (mean = 1043 cm) than those injected with saline (mean = 462 cm). Performance improved over the two test days, $F(1,34) = 70.26$, $p < 0.001$, and over blocks, $F(3,102) = 50.91$, $p < 0.001$, with smaller improvements across blocks on the second day (mean $\Delta = 581$ cm) when compared with the first day (mean $\Delta = 377$ cm), as indicated by a significant day × block interaction, $F(3,102) = 3.06$, $p < 0.05$. A significant day × block × dose interaction, $F(3,102) = 9.74$, $p < 0.001$, was attributable to much smaller improvements over blocks in the drugged animals, especially on day 1. With respect to swim speed, those rats administered the 5.6-mg/kg dose of CDP swam significantly slower (mean = 26.45 cm/s) than those injected with saline (mean = 29.55 cm/s, $F(1, 34) = 8.84$, $p < 0.01$. Swim speed declined across blocks, $F(3, 102) = 21.20$, $p < 0.001$, but increased from day 1 to day 2, $F(1,34) = 5.07$, $p < 0.05$. There were also significant block × dose, $F(3,102) = 21.20$, $p < 0.001$, and day × dose, $F(1,34) = 8.24$, $p < 0.01$, interactions.

Consistent with the probe trial of Experiment 2, the distance traveled in the target quadrant (percentage of total distance traveled) indicated a selective search of that quadrant for all groups (i.e., the distance traveled in the target quadrant was greater than that in each of the other three quadrants, $p < 0.05$; data not shown). For this measure, the two nutritional groups of rats did not differ in their dose response, although there was a significant overall dose effect, $F(1, 34) = 23.16$, $p < 0.001$, attributable a lower amount of searching in the target

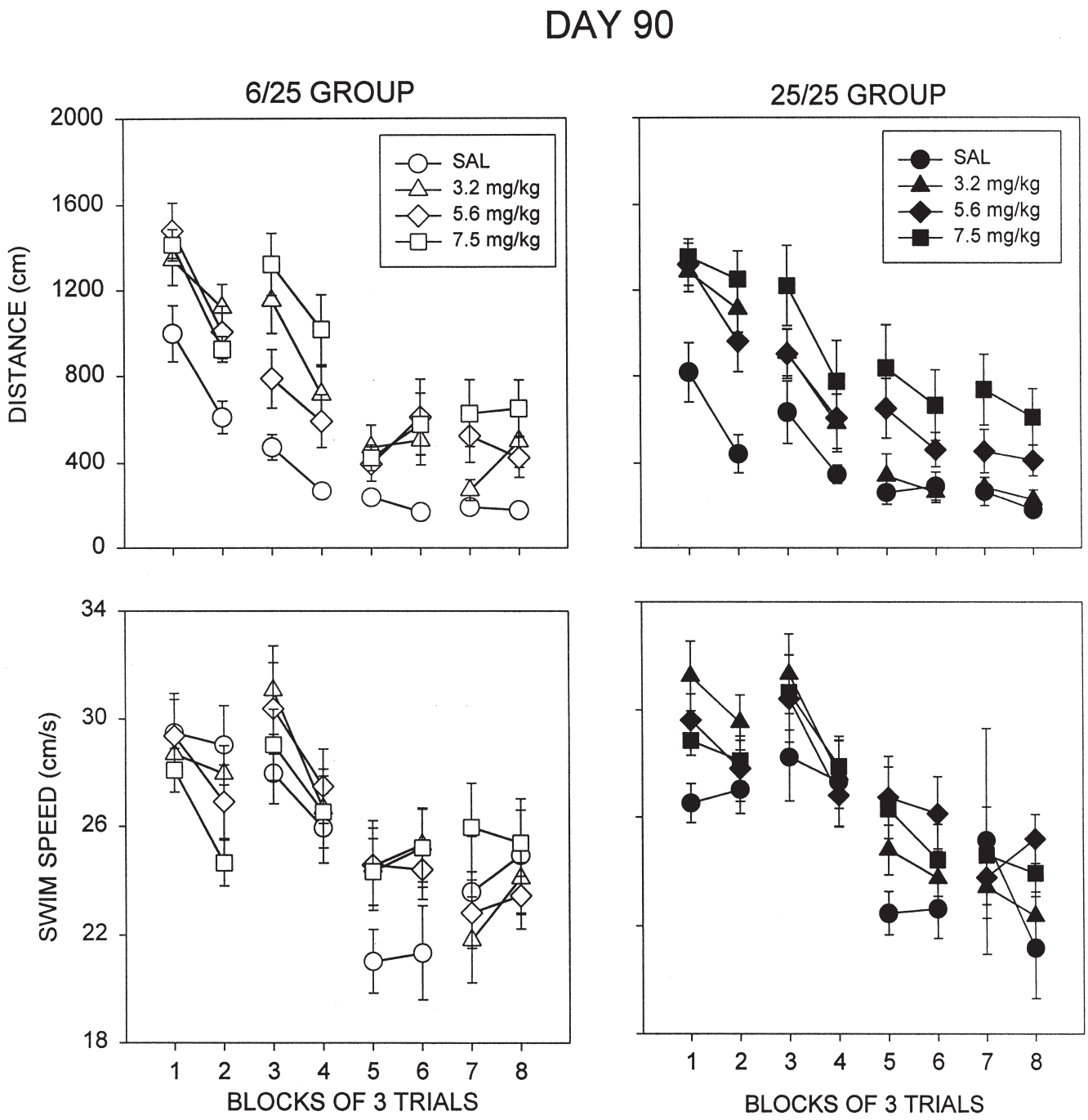

FIG. 3. Experiment 2: effect of CDP on acquisition of the Morris maze in adult 6/25 (left panels) and 25/25 (right panels) rats. Upper panels indicate the distance swum to the platform. Lower panels indicate the swim speed.

quadrant under CDP (saline = 49.8%, CDP = 33.5%). However, a differential dose response in the 6/25 and 25/25 nutritional groups was established upon analysis of annulus crossings (Fig. 6B). With the exception of the 25/25 5.6-mg/kg CDP group, all groups showed a significant difference in the number of annulus crossings between the four quadrants. In all of these cases, this finding was due to a significantly greater number of annulus crossings in the target quadrant relative to any in the other three quadrants ($p < 0.05$). Hence, after administration of a 5.6 mg/kg dose of CDP, selective and localized search patterns were completely destroyed in the 25/25 group, but retained in the 6/25 group. This differential dose effect was further confirmed by comparing the number of annulus crossings in the target quadrant between the 6/25 and 25/25 groups across dose conditions (i.e., saline or CDP). A significant nutritional group $\times$ dose interaction, $F(1,34) = 11.95$, $p < 0.01$) indicated that, under a 5.6-mg/kg dose of CDP, 6/25 rats showed significantly higher levels of annulus crossings in the

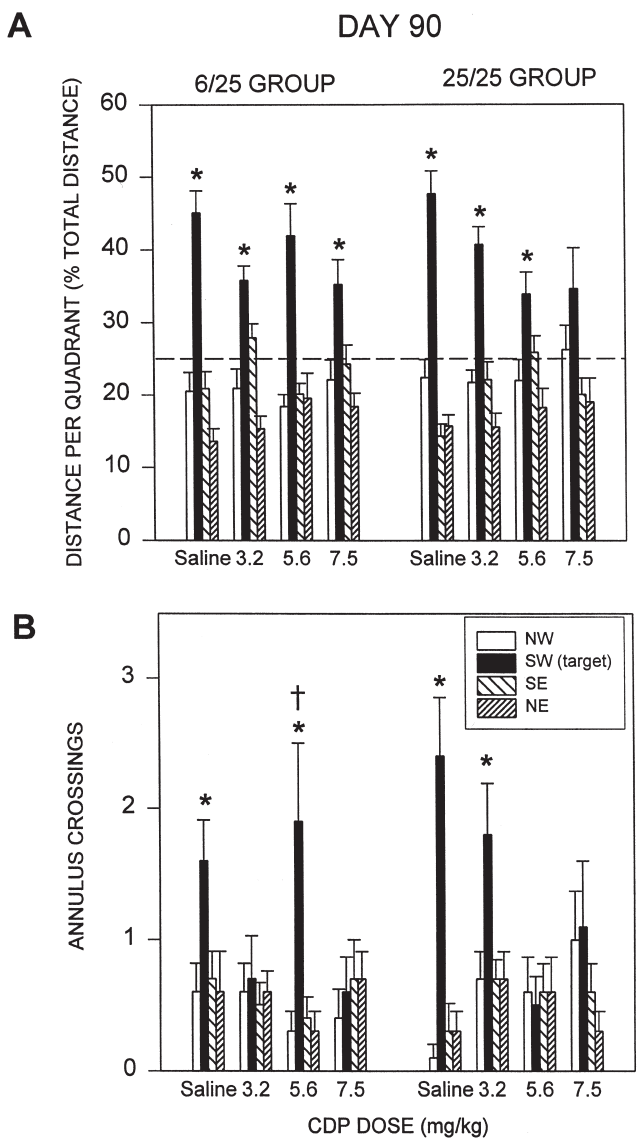

FIG. 4. (A) Experiment 2: effect of CDP on the relative distance traveled per quadrant in adult 6/25 and 25/25 rats during the probe trial (platform removed). *Target quadrant greater than chance level (indicated by dashed line), $p < 0.05$. (B) Experiment 2: number of annulus crossings in adult 6/25 and 25/25 rats during the probe trial. *Number of target annulus crossings significantly greater than the number of crossings of any of the other three quadrants ($p < 0.05$). †Target quadrant for the 6/25 group greater than that of the 25/25 group for the same dose of CDP ($p < 0.05$).

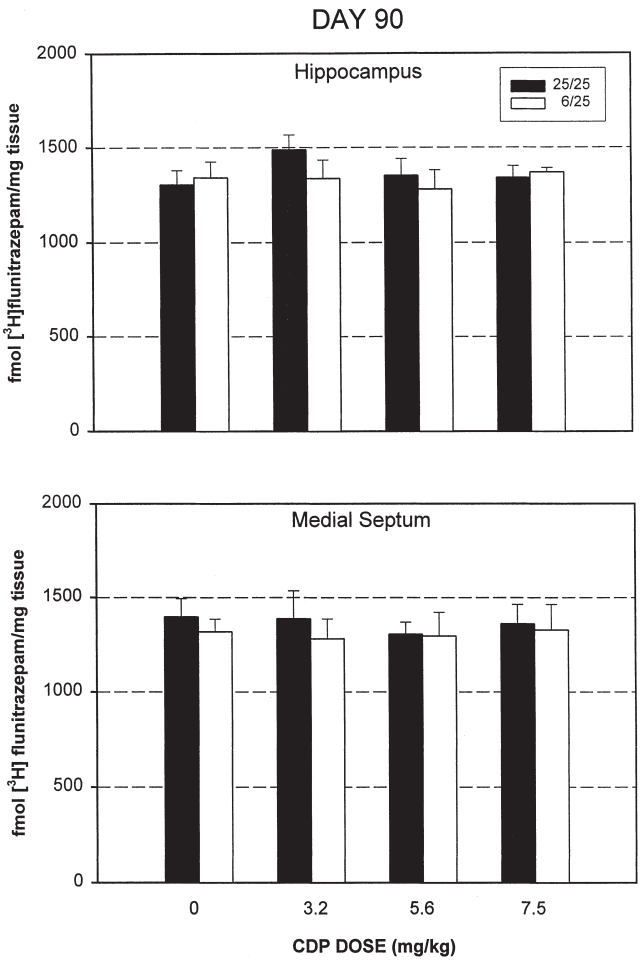

FIG. 5. Experiment 2: effect of prenatal malnutrition on [^{3}H]flunitrazepam binding within the hippocampal formation and the medial septum. Note the similarity of binding across both nutritional groups and across the CDP dose groups.

target quadrant than 25/25 rats ($p < 0.05$). Interestingly, in this cohort of rats, the 25/25 rats showed significantly more annulus crossings in the target quadrant than the 6/25 rats following saline injection ($p < 0.05$).

DISCUSSION

The present investigation demonstrates that rats with prenatal protein malnutrition display an altered amnestic response to benzodiazepine modulation of the $GABA_A$ receptor that is dose dependent and developmentally specific. At 30 days of age, prenatally malnourished rats showed a lower sensitivity than well-nourished controls to systemic application of the 5.6-mg/kg dose of CDP, indicated by significantly shorter distances traveled during acquisition and a more selective search of the target quadrant upon removal of the platform (probe trial). At 90 days of age, prenatally malnourished rats again showed a lower sensitivity to the 5.6-mg/kg dose of CDP, indicated by a greater number of target annulus crossings on the probe trial relative to the well-nourished controls. At this age, however, prenatally malnourished rats also exhibited greater sensitivity than well-nourished controls to the 3.2-mg/kg dose of CDP. The highly selective search pattern displayed by the controls on the probe trial was in sharp contrast to the nonselective search pattern exhibited by the prenatally malnourished rats—a finding that proved to be reliable in an independent replication. Interestingly, these group differences in the adult behavioral response to CDP were not correlated with a change in the number of BZ binding sites in either the hippocampus or the medial septum (Fig. 5).

The ability of prenatally malnourished animals to form and utilize a spatial strategy at a dose of CDP (~5 mg/kg) that has been firmly established as amnestic in the Morris water maze (28,29) is striking, and suggests a reduced sensitivity to the drug's amnestic properties, at this dose. Doses of CDP other

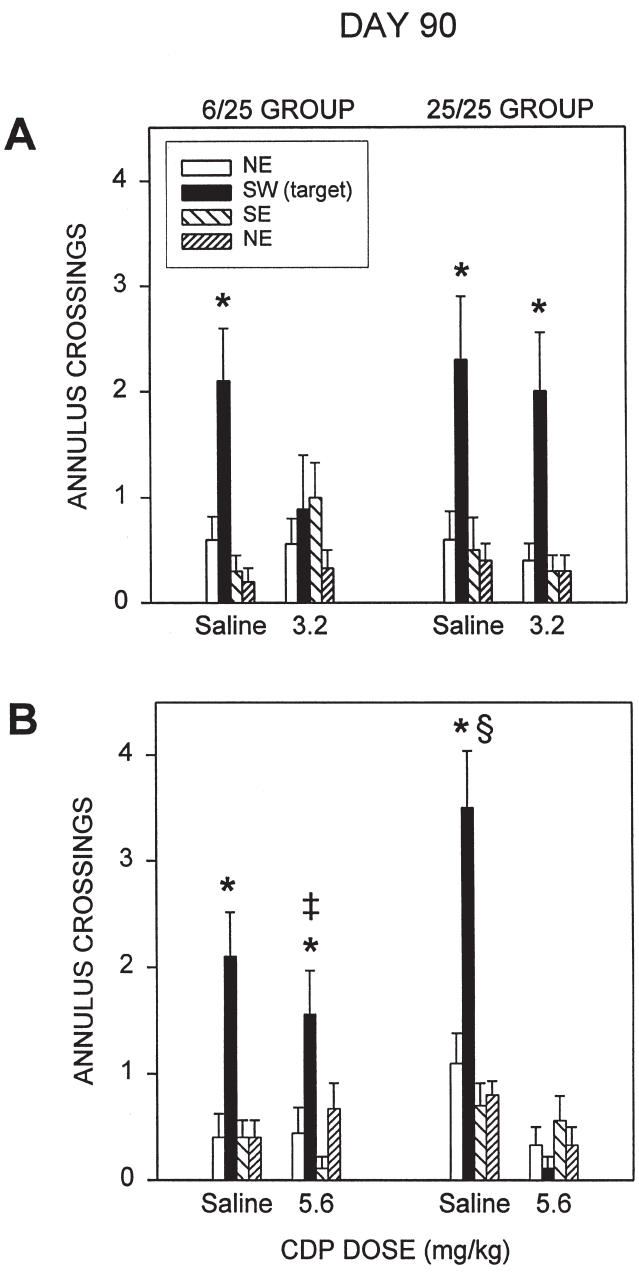

FIG. 6. Experiment 3: number of annulus crossings in adult 6/25 and 25/25 rats during the probe trial (platform removed) following (A) 3.2 mg/kg CDP, or (B) 5.6 mg/kg CDP. *Number of target annulus crossings greater than the number of crossings of any of the other three quadrants, within the same group ($p < 0.02$). ‡Target annulus crossings for the 6/25 5.6-mg/kg CDP group greater than that of the 25/25 5.6-mg/kg CDP group ($p < 0.05$). §Target annulus crossings for the 25/25 saline group greater than that of the 6/25 saline group ($p < 0.05$).

than 5 mg/kg (IP) have not been tested in previous learning paradigms using malnourished animals (2,5,13), most likely because the amnestic effects of lower doses of CDP are observed less consistently, and higher doses will also possess a powerful anxiolytic (18) and possibly sedative effect. The finding that CDP had no significant effect upon spatial learning performance in the well-nourished animals at the 3.2 mg/kg dose, confirms that this dose is not normally amnestic in the water maze test. It is intriguing therefore, that by day 90 prenatally malnourished animals developed a sensitivity to the 3.2-mg/kg dose of CDP, which served to eliminate their use of a spatial strategy (an effect not seen at day 30), whereas their spatial abilities were relatively unaffected by 5.6 mg/kg CDP, indicating an apparent insensitivity to the higher dose. Two previous studies utilizing shock-motivated avoidance procedures have also documented a reduced sensitivity of postnatally malnourished animals to a 5-mg/kg dose of CDP (2,5). In an inhibitory avoidance task the latency to step down from a raised platform onto a shock grid was measured following delivery of one footshock 30 min subsequent to CDP (5 mg/kg IP) or saline injection (2,5). When tested at postnatal day 49 or 70, well-nourished rats given CDP showed a significant reduction in step-down latency compared with those receiving saline. In contrast, rats provided with a low protein diet (8% casein) from birth to 49 days of age exhibited identical step-down latencies between those treated with CDP and those treated with saline (at both ages). Although the authors interpreted these findings as due to a reduced anxiolytic effect of the drug, it is clear that this dose of CDP also possesses amnestic properties in normal animals. Because the avoidance test incorporates a learning component, the findings can be alternatively interpreted as due to a reduced sensitivity to the amnestic properties of the 5-mg/kg dose of CDP in the malnourished animals, in accord with our findings. To our knowledge, the only learning study that employed motivation other than footshock (13) also documented lack of an amnestic response in malnourished animals to CDP when administered at a dose of 5 mg/kg IP. Whereas the drug clearly impaired the learning of a thirst-motivated Hebb–Williams maze task in the well-nourished animals, postnatally malnourished rats actually committed significantly fewer errors under CDP compared with those injected with saline.

Baseline differences in learning performance between the nutritional groups (as in the latter study) can serve to complicate interpretation of an altered rate of learning following a drug. In the current investigation baseline differences were not generally present. The only exception was in Experiment 3 (during the replication of the 5.6-mg/kg CDP dose findings) where, in the absence of the drug, the well-nourished group exhibited a more selective search pattern than the malnourished group on the probe trial. However, this difference in baseline performance did not affect the outcome because the findings of Experiment 3 were identical to those shown in Experiment 2, in which baseline levels were similar across groups.

Before considering the potential mechanisms by which prenatal malnutrition could promote differences in the behavioral response to CDP, we must consider the possibility that these effects are somehow related to differences in the way the drug is distributed, metabolized, or eliminated. Given the systemic route of drug administration, these considerations are paramount. The fact that the prenatally malnourished adult animals demonstrated both a greater sensitivity and a lower sensitivity to the drug at day 90, depending upon the dose, tends to argue against an orderly pharmacokinetic difference across the groups (at this age). Moreover, previous studies on perinatally protein malnourished rats have reported equivalent brain and plasma levels of BZs after acute systemic application (8). In our animal model, however, either (a) direct assessment of the brain levels of the drug and its active metabolites following systemic application, and/or (b) the central application of the drug, would be necessary to elimi-

nate the possibility of an altered BZ disposition, entirely. Of these two approaches we have chosen to focus our ongoing investigations on the behavioral response of prenatally malnourished animals to direct central application of drugs. When pharmacokinetic variables are controlled in this way, our preliminary findings (42) indicate that the prenatally malnourished rats appear less sensitive than well-nourished rats to equivalent levels of CDP administered directly in the brain, suggesting that an explanation for the present results must look beyond simple pharmacokinetic differences between the two groups.

Based upon the literature, we propose two possible mechanisms that could underlie the present results. First, prenatal malnutrition may alter subunit gene expression leading to the synthesis of different populations of $GABA_A$ receptors with varying affinities for BZs, or second, prenatal malnutrition may alter the allosteric interactions between the benzodiazepine site and the GABA site in populations of $GABA_A$ receptors that are otherwise similar. The protein complex that comprises the $GABA_A$ receptor consists of five subunits, grouped into at least six classes (α,β, γ, δ, ϵ, π) according to the degree of amino acid identity, with over 17 genetically distinct subtypes having been identified [reviewed in (34)]. Consequently, there is the capacity for enormous heterogeneity in the types of $GABA_A$ receptors produced in the developing and mature brain. The combination of subunits is known to greatly influence the pharmacological characteristics of the $GABA_A$ receptor (e.g., (32,33)]. Hence, even minor alterations in subunit gene expression consequent to prenatal malnutrition could generate different populations of $GABA_A$ receptor at the cell surface (with different dose–response characteristics to BZs). It has also been established that developmental changes occur in the mRNA coding for the various $GABA_A$ subunits (23) and that the $GABA_A$ receptors expressed at different stages of development (including the period 28–35 days to 45–52 days) differ in their pharmacological properties (21). Thus, the differences in response to CDP observed from 30 to 90 days of age following prenatal malnutrition may be due to a different developmental pattern for subunit gene expression than that present in the controls. The second possible mechanism underlying the present results is that prenatal malnutrition may bring about a change in the allosteric interactions between the BZ binding site and the GABA binding site on the $GABA_A$ receptor. Indeed, there is strong biochemical evidence to support the conclusion that allosteric interactions between GABA and BZ are significantly modified consequent to perinatal protein malnutrition (8). Potentiation of GABA-mediated chloride uptake in cerebral cortex microsacs with acute exposure to diazepam proved to be lower in rats with perinatal protein malnutrition than in well-nourished controls. This result would suggest that the protein restriction early in life diminishes the ability of benzodiazepines to modulate the GABA site on the $GABA_A$ receptor complex. Other prenatal insults, for example, ethanol, have also been shown to alter the modulation of the $GABA_A$ receptor (1). Both positive and negative neuromodulation of $GABA_A$ receptor-gated chloride flux was measured in membrane vesicles prepared from adult rats with prenatal ethanol exposure. Dramatic differences in the GABA-stimulated Cl^- flux in response to flunitrazepam and FG-7142, as well as alphaxalone and pregnenolone, were observed relative to controls, with the magnitude and direction of these effects being highly specific to the brain region examined.

In summary, the present findings provide additional support for the notion that malnutrition experienced early in development alters the future sensitivity to benzodiazepines, a result that suggests fundamental changes within the GABAergic system. Future experiments will be aimed at determining whether the alterations in $GABA_A$ receptor function, as monitored by our behavioral assay, are due to either an alteration in the synthesis of $GABA_A$ receptors, reflecting differential gene expression of the subunits that form the receptor, or due to a difference in the allosteric interaction between the benzodiazepine and GABA binding sites on an equivalent population of $GABA_A$ receptors.

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