ELSEVIER

Pharmacology, Biochemistry and Behavior 73 (2002) 759–767

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

www.elsevier.com/locate/pharmbiochembeh

Prenatal protein malnutrition enhances stimulus control by CDP, but not a CDP/THIP combination in rats

Penny L. Shultz*, Janina R. Galler, John Tonkiss

The Center for Behavioral Development and Mental Retardation, Boston University School of Medicine, M923 715 Albany Street, Boston, MA 02118, USA

Received 17 January 2002; received in revised form 20 May 2002; accepted 21 May 2002

Abstract

In the present study, the effects of prenatal protein malnutrition on stimulus control exerted by the benzodiazepine (BZ), chlordiazepoxide (CDP) and the GABA-A receptor agonist 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP) were characterized. The adult, male offspring of female Sprague–Dawley rats fed either low (6% casein) or adequate (25% casein) protein diets 5 weeks prior to mating and throughout pregnancy served as subjects. Subjects were first trained to discriminate CDP (8.0 mg/kg ip) from saline using drug discrimination procedures. Once a criterion level of performance was achieved, generalization tests were performed to lower doses of CDP (4.0, 2.0, 1.0, 0.5 and 0.25 mg/kg) and then to several doses of THIP (10.0, 7.5, 5.6 and 3.2 mg/kg). Lastly, the ability of a single dose of THIP (3.0 mg/kg) to enhance discriminative control by several low doses of CDP (4.0, 2.0, 1.0 and 0.5 mg/kg) was assessed. Although both diet groups acquired the original CDP/saline discrimination at the same rate, malnourished rats exhibited significantly more generalization to low doses of CDP than their well-nourished counterparts. Neither diet group exhibited significant generalization to THIP nor a difference in THIP's ability to enhance the CDP cue. These results suggest that a subject's sensitivity to the stimulus properties of drugs can be selectively modified by prenatal malnutrition.

Keywords: CDP; THIP; Protein malnutrition; Drug discrimination; Stimulus control; Rats

1. Introduction

Malnutrition, during both pre- and postnatal developmental stages in rodents, has been shown to significantly alter a subject's sensitivity to a variety of drugs acting through different neurotransmitter systems (Almeida et al., 1996; Butler et al., 1994). Most studies, however, have focused on the malnourished subject's behavioral response to psychoactive compounds that affect the GABA-A/benzodiazepine (BZ) receptors. Regardless of whether the malnutrition occurs prenatally, postnatally or both, most of these studies have reported a reduced sensitivity to the anxiolytic properties of several types of BZ receptor agonists (Almeida et al., 1988, 1990, 1991, 1992; Brioni and Orsingher, 1988; Brioni et al., 1989).

The effects of prenatal protein malnutrition on both the brain and behavior have been studied extensively in our laboratory using a rodent model (Galler et al., 1996; Tonkiss et al., 1993). These studies have provided evidence that prenatal protein malnutrition significantly alters the functioning of the GABAergic system, especially in the hippocampus. The functional consequences of these alterations have been characterized by evaluating the amnestic properties of the nonselective BZ receptor agonist, chlordiazepoxide (CDP), using the Morris water maze. When compared with well-nourished controls, adult, prenatally protein malnourished rats were less sensitive to the amnestic properties of a systemically administered moderate dose (5.6 mg/kg) of CDP, but more sensitive to a lower dose (3.2 mg/kg) of this same compound (Tonkiss et al., 2000a). Even when CDP was infused directly into the medial septum, an area of the brain critically involved in the production of BZ-induced amnesia (McNamara and Skelton, 1993), prenatally malnourished subjects exhibited a significantly reduced amnestic response to the 30- and 60-nmol doses (moderate and high), and a trend towards an enhanced response to the 15-nmol dose (low) (Tonkiss et al., 2000b).

In addition to their amnestic properties, BZs have other effects (anxiolytic, sedative, disinhibiting, anti-epileptic)

* Corresponding author. Tel.: +1-617-638-4755; fax: +1-617-638-5890.

E-mail address: pshultz@bu.edu (P.L. Shultz).

0091-3057/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0091-3057(02)00907-3

that are thought to be mediated either by different brain regions and/or by different configurations of the GABA-A receptor (Rudolph et al., 1999; Sieghart, 2000). Thus, how malnutrition alters a subject's sensitivity to one aspect of CDP's effect cannot necessarily be generalized to CDP's other properties. CDP produces a robust, easily discriminable cue in a drug discrimination paradigm (Sanger, 1987) that has been shown to be mediated through CDP's interactions with BZ receptors (De Vry and Slangen, 1986). Therefore, one aim of the present study was to investigate whether the differential sensitivity exhibited by prenatally protein malnourished rats to the amnestic properties of CDP was also exhibited when the stimulus properties of the drug were characterized using a standard two-lever, food reinforced drug discrimination procedure. If alterations in sensitivity to the stimulus properties of CDP are revealed in prenatally malnourished animals, this finding would lend support to the hypothesis that malnutrition's effect on the functioning of the GABA system is more extensive than previously acknowledged.

The drug discrimination paradigm has been used extensively to characterize the stimulus properties of drugs from a wide range of drug classes. These stimulus properties are very stable over multiple test sessions (Schechter et al., 1989) and, perhaps more importantly for investigations into the functional consequences of prenatal malnutrition, their intensity has been positively correlated with the drug's receptor binding affinity in the brain (Sanger and Benavides, 1993; Young and Glennon, 1987). During drug discrimination testing, subjects are taught to discriminate between the presence and absence of a training drug-related cue and respond appropriately (e.g., drug = right-lever, saline = left-lever). Once stimulus control by the training drug is established, generalization testing (a.k.a. substitution), either to other doses of the training drug or to completely different drugs, can begin. Drugs that are pharmacologically similar to the training drug tend to produce responding on the training drug-associated lever (generalization), while drugs with pharmacologically disparate modes of action do not (Ator and Griffiths, 1986, 1989). This degree of specificity has made drug discrimination a valuable tool for characterizing the commonalities of subjective effects within a drug class and for establishing the functional relevance of a particular drug class' mode of action.

The GABA-A receptor has been shown to modulate the intensity of the discriminative cue produced by BZs. Nielsen et al. (1983) has reported that although the direct GABA-A receptor agonist, 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP), only minimally generalized to the cue produced by the BZ receptor agonist diazepam (DZ) during drug discrimination testing, THIP was able to significantly enhance the DZ cue when the two drugs were administered together. Consequently, it is possible to assess the functional interactions between two distinctly different sites on the GABA-A receptor complex using drug discrimination procedures. Such interactions, which would provide important additional information regarding how malnutrition may alter the functional interactions within the GABA-A supramolecule complex, have not been assessed in animals following prenatal protein malnutrition. This has made it difficult to determine whether the changes in BZ agonist sensitivity exhibited by malnourished animals were mediated by alterations in the BZ receptor itself (e.g., affinity), or if they were the result of functional changes in the interactions between the BZ and GABA-A binding sites. Thus, an additional aim of the present study was to characterize the amount of generalization the prenatally malnourished animals exhibited to THIP following training with CDP, as well as THIP's ability to enhance the stimulus produced by low doses of CDP.

2. Methods

2.1. Nutritional treatment

Virgin, female, Sprague–Dawley rats were obtained from Charles River Laboratories (Kingston, MA). One group was placed on an adequate protein diet (25% casein, Teklad Laboratories, Madison, WI) 5 weeks prior to mating and throughout pregnancy, while another group was fed an iso-caloric, low protein diet (6% casein, Teklad Laboratories) throughout the same time period. All females were mated with males that had been acclimated to these respective diets for 1 week. Throughout pregnancy, dams were singly housed in polycarbonate breeding cages measuring $51 \times 41 \times 21$ cm (Lab Products, Maywood, NJ). Following parturition, all litters were culled to eight pups (two females and six males) and cross-fostered as whole litters to females of the 25% casein diet group that had given birth within the same 24 h period. Pups born to mothers on the 6% casein diet that were fostered to mothers on the 25% casein diet were designated as members of the 6/25 (prenatally malnourished) group, while pups born to mothers on a 25% casein diet that were also fostered to other mothers on a 25% casein diet were designated as members of the 25/25 (prenatally well-nourished) group. At Day 21, all rats were weaned and placed on a standard laboratory chow diet (Purina Mills, Richmond, IN; Formula 5001).

2.2. Subjects

One male rat from each of 15, 6/25 litters and 15, 25/25 litters served as subjects. Subjects were approximately 70 days old at the start of testing and were individually housed in polycarbonate micro-isolator cages ($47 \times 25 \times 20$ cm) throughout the study. The colony rooms were maintained at 73 ± 3 °F with 45–55% humidity and were kept on a 12:12-h reverse light/dark cycle with lights on at 19:00 h. During the dark part of the cycle, red florescent lighting provided dim illumination. Sessions were conducted once a day, 5 days/week, and all testing occurred during the dark

phase of the cycle, which corresponded with the active waking period of the rats. The subjects were given daily food rations that gradually reduced them to 85% of their free-feeding body weight, and were maintained at this weight throughout the course of the study by careful daily food rationing. They were given free access to water in their home cages. All procedures described in this paper were approved by the Boston University Medical School Institutional Animal Care and Use Committee (Approval #01-057) and follow guidelines outlined in *Guide for the Care and Use of Laboratory Animals* (NIH publication #97-23).

2.3. Apparatus

Sessions were performed using ten, operant test chambers ($32 \times 25.5 \times 25.5$ cm) enclosed in sound-attenuated boxes with ventilation fans (Med-Associates, St. Albans, VT, USA). Each chamber was outfitted with two retractable response levers mounted 11 cm apart with associated stimulus light. A house light, located at the top of the back panel of each operant chamber, provided ambient illumination during a test session. A pellet dispenser delivered the 45-mg pellets that served as reinforcers (Dustless Precision Pellets, Bio-Serv, Frenchtown, NJ) into a food hopper, located halfway between both response levers. An IBM-clone Pentium computer programmed with MED-PC for Windows software (version 1.17, Med-Associates) controlled experimental sessions and data collection.

2.4. Drugs

CDP HCl and THIP HCl were purchased from Sigma/Aldrich (St. Louis, MO, USA). Both drugs were dissolved in a 0.9% saline solution to form all the test compounds. This saline solution was also administered alone during saline training sessions. All drugs were injected intraperitoneally in a volume of 1 ml/kg to achieve the following doses: for CDP: 8.0, 4.0, 2.0, 1.0, 0.5 and 0.25 mg/kg and for THIP: 10.0, 7.5, 5.6 and 3.2 mg/kg. All drug solutions were made fresh daily.

2.5. Procedures

2.5.1. Preliminary training procedure

All training and testing sessions, given once per day, were 15 min in duration and were conducted 5 days/week. Subjects were first trained to respond on each lever such that a lever press yielded a single reinforcer (CRF), and then gradually shaped to respond on both response levers on a fixed-ratio 10 (FR 10) schedule of reinforcement. Once all the subjects were responding on both levers with equal proficiency (10 days), discrimination training began. Half of the rats from each nutritional group were assigned to operant chambers with a drug-right/saline-left lever assignment, while the remainder were assigned to chambers with the opposite lever assignment. Subjects were then trained to discriminate between an intraperitoneal injection of the training dose of CDP (8.0 mg/kg), a dose of CDP that produces a robust, easily discriminable cue without producing a significant reduction in response rate, and an intraperitoneal injection of saline.

On each training day, subjects were injected with either the training dose of CDP or saline and then 15 min later, placed in their respective operant chambers. Injections were administered using the following pseudo-random sequence: DSDSD, DSSDS, SDSDS, SDDSD where S = a saline day and D = a drug day. Subjects were given three choice trials during a session. This method of drug discrimination training (as described in Tomie et al., 1995) was chosen because it has been shown to help maintain discriminative control by CDP despite repeated low dose testing, and it significantly enhances the rate of acquisition of the drug discrimination task.

At the beginning of each of the three trials within the session, the house light was illuminated and both left and right levers were extended. If the subject made a correct choice by completing 10 responses on the injection appropriate lever before completing 10 responses on the alternate lever, the subject was rewarded with a 45-mg pellet. Moreover, that same lever remained extended, providing the subject with nine more opportunities to procure the food reward on an FR 10 schedule. If, however, subjects chose the incorrect lever, they were not reinforced and the lever remained extended for a 5-min extinction period. Following a 30-s intertrial-interval during which time the chambers were dark, the second of three discrimination choice trials was executed. The procedures used in the second and third choice trials were identical to the first.

Only data from the first trial of the session was evaluated. These data consisted of first trial choice, the total number of first trial responses, the percentage of injection appropriate first trial responses and the first trial choice latency. First trial choice was defined as the assignment of the lever that the subject completed 10 responses on before completing 10 responses on the alternative lever. The total number of first trial responses was defined as the total number of responses the subject made on both levers. The percentage of injection appropriate first trial responses was defined as the percentage of the total number of the responses that the subject made on the injection appropriate lever. The first trial choice latency was defined as the number of seconds that elapsed between initiation of the session and the subject's completion of 10 responses (a choice) on one of the levers.

2.5.2. Generalization (substitution) testing procedure

Once the subjects demonstrated a criterion level of stimulus control by the training drug, generalization-testing sessions commenced. Criterion performance was considered to have been achieved for each subject when their first trial percent of injection appropriate responses reached 90% or better for 9 of 10 consecutive training sessions. There were

three, 25/25 subjects that were dropped from further participation in the study because they were either unable to demonstrate equal proficiency on both levers when responding on an FR 10 schedule of reinforcement ($n = 1$) or were unable to learn the original discrimination despite several month of training ($n = 2$). On the average, criterion performance was achieved by both nutritional groups in about 20 (range = 11–30) sessions and generalization testing began approximately 3 days after all remaining subjects had achieved criterion performance.

Generalization testing sessions were conducted in a manner similar to the training sessions except for the following two procedural changes: (1) subjects received only one trial during each session instead of three and (2) a choice of either lever resulted in reinforcement. These alterations were made to limit the subject's experience with doses and drugs other than the training drug during generalization testing. The drug being tested determined the time interval between injections and initiation of a test session. CDP generalization testing sessions were initiated 15 min following an intraperitoneal injection of the drug, while THIP testing sessions occurred 30 min following the drug injection. Doses of each of the test drugs were administered in a pre-assigned, random order and the same sequence of test sessions was used for each subject. CDP generalization testing was completed first followed by THIP generalization testing. Generalization tests were conducted once a week, and intermixed with saline and training drug sessions with at least one saline and one drug training session between each generalization testing session to confirm that the criterion level of discriminative control by the training drug was being maintained.

2.5.3. THIP/CDP interactions

Lastly, tests were performed which measured the extent to which a single dose of THIP (3.0 mg/kg) increased the subject's amount of generalization to relatively low doses of CDP. First, to re-evaluate the amount of drug-appropriate responding the subjects were exhibiting to relatively low doses of CDP following the THIP generalization testing outlined above, the dose–response curve for this drug was redetermined. The generalization testing procedure that was used was very similar to that used during the original generalization testing. However, the test doses were administered 30 min prior to the initiation of a test session rather than the original 15-min time period (in preparation for the next part of the study where THIP, which takes longer to have its peak effect, would be administered in conjunction with CDP), and the test doses of CDP that were evaluated were only the 0.5, 1.0, 2.0 and 4.0 mg/kg doses. Once again, generalization tests were performed once per day and were interspersed among training days.

Once the CDP dose–response curve had been redetermined, tests were performed to assess the ability of THIP to enhance generalization to low doses of CDP in all subjects. On each test day, subjects were injected intraperitoneal with a combination of THIP (3.0 mg/kg) and one of the four possible test doses of CDP (0.5, 1.0, 2.0 and 4.0 mg/kg). The doses of CDP were tested in a pre-assigned, random order and all subjects received all doses over the course of generalization testing. Thirty minutes later, subjects were placed in their respective operant chambers and testing procedures were initiated. Once again, subjects received only one trial per session and a choice of either lever resulted in reinforcement.

2.6. Data analysis

The effects of the prenatal nutritional treatment on body weight at birth and at the start of the study (Day 70) were analyzed using one-way ANOVAs with prenatal nutritional treatment (6/25 vs. 25/25) as the independent variable. Drug discrimination acquisition and generalization test data were analyzed separately. To examine possible differences in the rate of acquisition of the original CDP/saline discrimination task, the mean percent responding on the injection appropriate lever and the mean first trial choice data (±S.E.M.) were calculated for each nutritional treatment group. These values were then used to ascertain the total number of training sessions required by each subject to achieve criterion performance. Total sessions were analyzed using a one-way ANOVA, with nutritional treatment (6/25 vs. 25/25) as the independent variable. To assess possible differences in the rate of responding during different types of training sessions, the mean choice latencies from the CDP and saline training sessions (in seconds) were calculated for both of the two prenatal treatment groups. These values were compared using a two-way ANOVA with nutritional treatment (6/25 vs. 25/25) and training session type (CDP vs. saline) as independent variables.

To compare the amount of generalization exhibited by the two nutritional groups to test doses of either CDP or THIP, the percent of responding on the CDP lever was calculated for each subject in each nutritional treatment group at each drug dose. These values were compared using two-way repeated measures ANOVA with nutritional treatment (6/25 vs. 25/25) as a factor, and dose (0.0, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/kg for CDP and 3.2, 5.6, 7.5 and 10.0 mg/kg for THIP) as the repeated measure. ED_{50} values (with 95% confidence limits) were calculated where appropriate using log doses and regression analyses. The effect of each type of test drug on response rate was determined by comparing the choice latency between the two nutritional treatment groups using two-way repeated measures ANOVA with nutritional treatment (6/25 vs. 25/25) as a factor and dose (0.0, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/kg for CDP and 3.2, 5.6, 7.5 and 10.0 mg/kg for THIP) as a repeated measure.

Finally, to compare the two nutritional treatment groups in the ability of THIP to shift their CDP generalization curves, the percent of responding on the CDP-lever during the CDP curve redetermination testing and during the CDP + THIP

combination testing were calculated for the subjects in each nutritional treatment group at each test dose. These values were then compared using a three-way repeated measure ANOVA with nutritional treatment (6/25 vs. 25/25) and test phase (CDP alone vs. CDP + THIP) as independent variables, and CDP dose (0.5, 1.0, 2.0 and 4.0 mg/kg) as a repeated measure. Alterations in choice latency values were also analyzed for this phase of the study using two-way, repeated measures ANOVA with nutritional treatment (6/25 vs. 25/25) as the independent variable and test phase (CDP alone vs. CDP + THIP) as a repeated measure.

3. Results

3.1. Weight data

Mean body weights ± S.E.M.s were compared at birth and at the start of the study (Day 70). At birth, the mean weight of the 6/25 pups (5.49g ± 0.23) was significantly lower then the weight of the 25/25 pups (6.49g ± 0.14) [$F(1,29) = 13.76$, $P < .001$]. By Day 70, however, the mean weights of the two groups were no longer significantly different (6/25 = 481g ± 9.6; 25/25 = 494g ± 12.9) [$F(1,29) = 0.695$, n.s.].

3.2. Acquisition data

Acquisition of the original CDP/saline discrimination required an average of 16.8 ± 1.0 (range = 12–24) sessions for the 6/25 subjects and 16.9 ± 1.6 (range = 11–30) sessions for the 25/25 subjects. This difference was not significant [$F(1,28) = 0.001$, n.s.]. By the end of the acquisition phase, both groups of animals were responding on the CDP-lever an average of 98% of the time following a CDP injection and only 9% of the time following a saline injection. Although subjects made a lever selection faster during training sessions where they had received a CDP injection, the average choice latencies of the two diet groups, within both CDP (6/25 = 9.5s and 25/25 = 8.2s) and saline (6/25 = 12.4s and 25/25 = 13.9s) training days, were similar. ANOVA confirmed that there was a significant main effect of the session type, with the subjects making a lever choice significantly quicker during CDP sessions than during saline training sessions [$F(1,56) = 9.5$, $P < .01$]. There was neither a significant effect of nutritional treatment on the latency to make a choice [$F(1,56) = 0.011$, n.s.] nor a significant interaction between nutritional treatment and training session type [$F(1,56) = 1.03$, n.s.].

3.3. CDP generalization data

Changing the dose of CDP given to subjects altered their percent of responding on the CDP-associated lever in a dose dependent and diet dependent way. The dose–response curves are illustrated in Fig. 1. Regression analysis of the

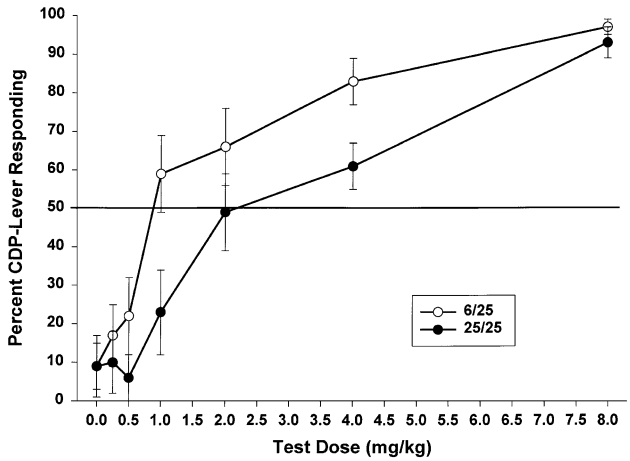

Fig. 1. The mean percentage of CDP-lever responding for 6/25 ($n = 15$) and 25/25 ($n = 12$) subjects across the seven test doses of CDP during generalization testing. Vertical bars represent the S.E.M.s for each test dose. The horizontal line at the 50% mark indicates the estimated ED_{50} test dose value for each diet group (6/25 = 1.64 mg/kg, 25/25 = 2.00 mg/kg). The 6/25 group exhibiting a significantly greater percentage of responding on the CDP-lever than the 25/25 group [$F(1,25) = 4.6$, $P < .05$].

CDP dose–response curve yielded an ED_{50} value of 1.04 mg/kg (95% CL = 1.01–1.97 mg) for the 6/25 diet group and an ED_{50} value of 2.00 mg/kg (95% CL = 0.9–2.22 mg) for the 25/25 diet group. Comparisons of the percent of CDP-lever responding between the two nutritional treatment groups revealed a significant main effect of dose [$F(4,100) = 17.9$, $P < .001$], indicating a significant general decline in the percent of CDP-lever responding as the amount of CDP administered decreased. There was also a significant diet effect [$F(1,25) = 4.6$, $P < .05$], with the malnourished group exhibiting a significantly greater percentage of responding on the CDP-lever than their well-nourished counterparts. No significant interaction between test dose and diet was revealed [$F(4,100) < 1$, n.s.]. When a similar analysis was performed on the choice latency data, there were no significant differences between the two nutritional treatment groups in their latency to make a choice, test dose did not significantly affect choice latency, and there were no significant interactions between these two factors.

3.4. THIP generalization data

For the most part, THIP failed to substitute for CDP in any of the generalization testing for both nutritional treatment groups (see Fig. 2). Even at the highest dose of THIP administered (10.0 mg/kg), only 2 of the 15, 6/25 subjects (13%) and 3 of the 12, 25/25 subjects (25%) chose the CDP-associated lever. When choice latency during the THIP generalization testing sessions was compared with choice latency during the interspersed CDP and saline training sessions, it was significantly increased for both the malnourished (increased by 422%) and control (591%) subjects [$F(1,6) = 10.87$, $P < .05$]. However, there was no significant

difference in the level of this increase between the malnourished and control animals [$F(1,6) = 0.385$, n.s] and no significant interactions were indicated by ANOVA [$F(1,6) = 0.407$, n.s.].

3.5. CDP redetermination curve data

In general, the subjects responded less on the CDP-lever during redetermination testing, with the largest decrease occurring at the lowest CDP test doses (for the 0.5 mg/kg dose, only 4% of responding was on the CDP-lever for the 6/25 subjects and 1% responding for the 25/25 subjects). Regression analysis performed on these data yielded an ED_{50} value of 2.6 mg/kg (95% CL = 2.05–3.15) for the 6/25 subjects and an ED_{50} value of 2.00 mg/kg (95% CL = 0.63–3.2) for the 25/25 subjects. These ED_{50} values represent an increase for the 6/25 subjects when compared with the original CDP dose–response curve ED_{50} values while the ED_{50} value for the 25/25 subjects was maintained. When a comparison was made between the choice latencies from the two diet groups during redetermination testing, there were no significant main effects or interactions.

3.6. THIP/CDP interaction data

The dose–response curves in Fig. 3 illustrate a leftward shift in the CDP curves for both the prenatally malnourished and well-nourished subjects when a set dose of THIP (3.0 mg/kg) was administered in conjunction with each test dose of CDP. It can be seen in this figure that this drug combination increased the mean percent of responding on the CDP-lever when compared with the level of responding using CDP injections alone. When the ED_{50} values were

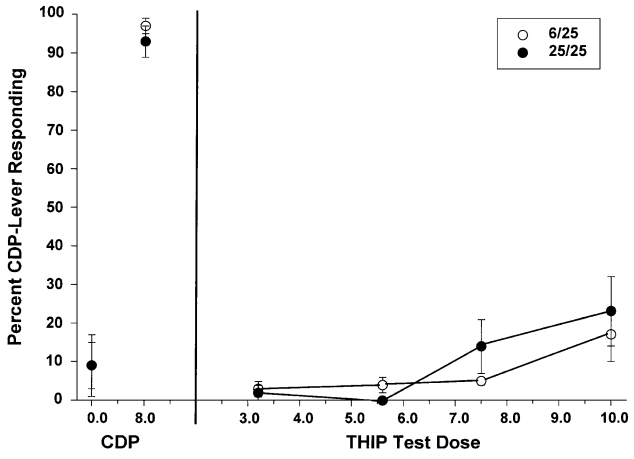

Fig. 2. The mean percentage of CDP-lever responding for the 6/25 ($n = 15$) and 25/25 ($n = 12$) subjects across the four test doses of THIP during generalization testing. Also shown are the mean percentages of CDP-lever responding that occurred during the CDP (8.0 mg/kg) and saline training sessions that were interspersed among THIP test sessions. Vertical bars represent the S.E.M.s for each test dose.

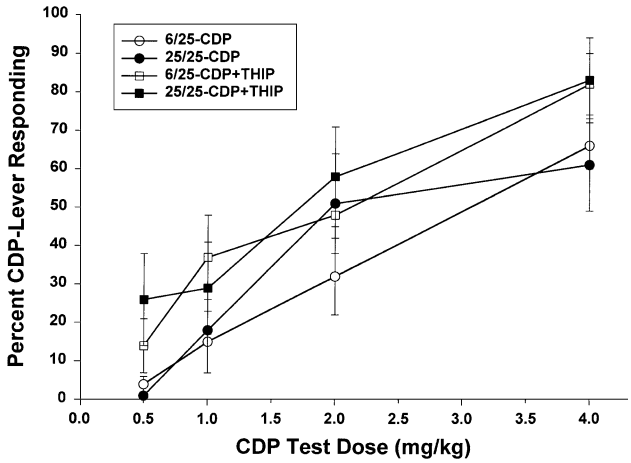

Fig. 3. The mean percentage of CDP-lever responding for the 6/25 ($n = 15$) and 25/25 ($n = 12$) subjects across the four test doses of CDP during both CDP redetermination testing (circles) and CDP + THIP generalization testing (squares). Vertical bars represent the S.E.M.s for each test dose. In both diet groups, the CDP + THIP combination producing significantly more responding on the CDP-lever than CDP alone [$F(1,56) = 4.47$, $P < .05$].

calculated for the CDP + THIP dose–response curves, an ED_{50} value of 1.9 mg/kg (95% CL = 0.9–2.93 mg) was revealed for the 6/25 diet group, while an ED_{50} value of 1.75 mg/kg (95% CL = 0.74–2.2 mg) was present for the 25/25 diet group. When the percent of CDP-lever responding was compared between the two diet treatment groups during the CDP-only phase of testing with the CDP + THIP phase, a significant main effect of drug treatment was revealed [$F(1,56) = 4.47$, $P < .05$], with the CDP + THIP combination producing significantly more responding on the CDP lever than CDP alone. There was also a significant effect of dose [$F(3,168) = 49.174$, $P < .001$], with the higher doses of CDP, with or without THIP, producing significantly more responding on the CDP lever than the lower doses of the drug. However, there was no significant difference in the percent of CDP-lever responding between the two prenatal diet groups, nor were there any significant interactions between the three variables.

On the average, choice latency was decreased during the CDP + THIP test sessions when compared with CDP-only sessions. This indicates that the subjects made a faster lever choice when administered the drug combination than when they were given CDP alone (decreased by 8.7% for malnourished subjects and by 6.5% for the well-nourished controls). This decrease in choice latency was not found to be significantly different between the two diet groups and there were no significant interactions.

4. Discussion

Results from the present study indicate that, using drug discrimination procedures, prenatally protein malnourished and well-nourished rats learn to discriminate a relatively

high dose of CDP from saline at about the same rate and with the same level of accuracy. However, the prenatally malnourished rats exhibited significantly more generalization to the stimulus properties of several lower doses of CDP than their well-nourished counterparts. Given that the level of discriminative control exerted by a drug is determined by the perceived robustness of the drug's cue, the present findings indicate that prenatal protein malnutrition increases the sensitivity of adult rats to the stimulus properties of CDP.

During the redetermination of the CDP dose–response curve, all subjects, especially in the malnourished group, exhibited a decrease in responding on the drug-appropriate lever. This decrease in responding resulted in a lessening of the difference between the malnourished and control subjects in their generalization to selected doses of CDP. There are a number of factors that could have produced this decrease. For example, several weeks had elapsed between generation of the first and the second CDP dose–response curves. Thus, the subjects were older the second time they were tested. It is possible that as the prenatally malnourished animals matured, compensations for the prenatal insult occurred that more closely equated these animals to the controls in their sensitivity to the CDP cue. While this could explain the decrease in drug-lever associated responding exhibited by the malnourished rats, it does not address why a decline in responding was also seen in the well-nourished rats, albeit to a lesser degree. A more likely explanation would be one common to both groups. It has been reported that the length of time following an injection of CDP can significantly alter the level of discriminative control exerted by the drug (Bronson and Chen, 1996). Thus, this decrease in discriminative control could also be the result of a decrease in the intensity of the CDP cue produced by the longer delay between the test dose injection and generalization testing. However, when CDP was tested within a time-frame that corresponded with its peak level of stimulus intensity, the malnourished subjects exhibited a greater sensitivity to the stimulus properties of the drug than the well-nourished controls.

These results are consistent with our previous finding that, at a dose which is comparable to the highest dose of CDP tested in the present study (3.2 mg/kg), adult, prenatally protein malnourished animals were more sensitive than well-nourished controls to the amnestic properties of CDP in a Morris water maze task (Tonkiss et al., 2000a). It is also interesting to note that in the same study, at a higher dose of CDP (5.6 mg/kg), the prenatally protein malnourished animals were actually *less* sensitive to the amnesic properties of CDP. This decreased sensitivity to CDP at doses of 5.0 mg/kg or higher has also been reported in other studies that have investigated CDP's anxiolytic and disinhibiting effects (Almeida et al., 1988, 1992). Prenatal malnutrition has been shown not have a uniform effect across all brain regions (Morgane et al., 1993). Furthermore, BZ's effects (anxiolytic, sedative, disinhibiting, antiepileptic and amnestic) can be mediated either by different brain regions and/or by different configurations of the GABA-A receptor (Rudolph et al., 1999; Sieghart, 2000). Thus, malnutrition's differential effects on sensitivity to CDP could at least in part be determined by which brain region the drug is primarily acting upon to produce the measured behavioral outcome, as well as the length and type of malnutrition employed.

Despite differences in the degree of generalization exhibited by the two prenatal treatment groups to low doses of CDP, there was no difference in their choice latency across the six test doses. This finding suggests that, at least within the range of doses tested, CDP's sedative properties did not hamper the rate of lever selection. Furthermore, if choice latency can also be interpreted as a measure of the level of confidence the animals had in their choice, then the present findings indicate that both nutritional treatment groups were equally certain about the correctness of their lever choice. Thus, at the lower doses of CDP, the well-nourished subjects were as certain that they had received a saline injection as the malnourished subjects were that they had not.

When the direct GABA-A receptor agonist, THIP, was administered during a testing session, both prenatal diet groups exhibited very little generalization to the CDP cue. This finding is consistent with those of Nielsen et al. (1983), who also reported a lack of generalization by THIP to the cue produced by another BZ receptor agonist (DZ). The present finding provides further evidence that, despite having a common final effect on chloride flux through the Cl-ionophore, GABA-A receptor agonists and BZ agonists produce distinctly different discriminative cues that do not readily cross-generalize. The meager amount of generalization exhibited by the subjects in the present study during THIP testing made it difficult to determine whether there was a sensitivity difference between the malnourished and control animals to THIP's stimulus properties. Subjects also took significantly longer to make a lever choice during THIP generalization testing. This provides additional evidence that subjects were uncertain what the injection appropriate lever was during the THIP test sessions. Clearly, the only way to determine whether prenatal malnutrition has a significant impact on the stimulus properties of direct GABA agonists will be to use these compounds as the training drug in future studies.

Although previous studies have characterized the sensitivity of malnourished animals to a variety of BZ agonists (see Almeida et al., 1996 for a review), to the best of our knowledge, the present study is the first to characterize how this sensitivity could be modified by a direct GABA-A receptor agonist. When compared to the amount of drug-lever responding produced by CDP alone, THIP, given in combination with CDP, significantly enhanced the amount of responding on the CDP-lever in both dietary treatment groups. Subjects also made a faster lever choice during the THIP + CDP sessions. Both findings indicate that THIP significantly enhance discriminative control by low doses

of CDP but did so to a similar degree in both prenatal diet groups. Teissere and Czajkowski (2001) have recently reported that GABA can produce a conformational change in the BZ receptor, which alters its affinity. If a GABA-A receptor agonist like THIP could also produce a similar conformational shift at the BZ receptor site, this shift could mediate the enhancement of CDP's discriminative stimuli seen in the present study. However, because the extent of the shift in the CDP curves was similar in both nutritional treatment groups, it would seem that the mechanism which enables THIP to modulate the discriminative stimulus properties of CDP is unaffected by the prenatal malnutrition insult. This is particularly interesting given the difference between the prenatally malnourished and well-nourished controls in their sensitivity to CDP alone. If taken together, these findings could indicate that malnutrition has its effect on CDP sensitivity by producing alterations at the BZ receptor level rather than by directly altering the GABA-A receptor itself. Sensitivity to the interoceptive cue produced by BZ agonists has been correlated with their binding affinities at BZ receptors, especially in the hippocampus (Sanger and Benavides, 1993). Thus, increased stimulus control by CDP in the malnourished animals can be interpreted as evidence of functional alterations in binding affinity rather than receptor number. In any case, previous studies in our laboratory have shown no difference in the density of BZ receptors in either the medial septum or the hippocampus of prenatally malnourished and well-nourished adult rats (Tonkiss et al., 2000b).

Although there is no data currently available to show the exact mechanisms by which prenatal protein malnutrition alters the GABA-A/BZ receptor complex, other prenatal insults, like chronic ethanol or cocaine exposure, have been shown to effect the functioning of both the BZ and GABA-A receptors. Prenatal ethanol exposure has been reported to decrease BZ receptor binding affinity in the cortex of adult animals (Bailey et al., 1999; but see Costa et al., 2000 for a review), while prenatal cocaine has been shown to increase the field potentials in GABA-A receptors in the hippocampus (Little and Teyler, 1998). If these prenatal insults can alter the functioning of the BZ/GABA-A receptor, it is also possible that prenatal malnutrition could alter this receptor complex in similar ways. To elucidate the mechanisms behind the present findings, a comprehensive analysis of the GABA/BZ receptor system in prenatally malnourished subject will need to be performed at the molecular level.

In summary, the present findings indicate that prenatal protein malnutrition enhances the ability of subjects to discriminate relatively low doses of CDP without affecting the ability of THIP to enhance the CDP cue. Because BZ's behavioral effects can be mediated by different brain regions or even by different configurations of the GABA-A receptor, the present findings confirm that malnutrition can alter more than one aspect of CDP's properties. Thus, these findings add to an increasing body of evidence that prenatal protein malnutrition significantly affects the functioning of the GABA/BZ receptor complex.

Acknowledgements

We would like to thank Michelle Vincitore, Kristin Bonnie and Elizabeth Haskins for their invaluable technical assistance throughout the study. This research was conducted with support from National Institute of Health grant no. HD22539 to J.R.G.

References

Almeida SS, de Oliveira LM, Bichuette MZ, Graeff FG. Early malnutrition alters the effect of chlordiazepoxide on inhibitory avoidance. Braz J Med Biol Res 1988;21:1033–6.

Almeida SS, de Oliveira LM, Graeff FG. Decreased reactivity to anxiolytics caused by early protein malnutrition in rats. Pharmacol, Biochem Behav 1990;36:997–1000.

Almeida SS, de Oliveira LM, Graeff FG. Early life protein malnutrition changes exploration of the elevated plus-maze and reactivity to anxiolytics. Psychopharmacology 1991;103:513–8.

Almeida SS, Soares EG, Bichuette MZ, Graeff FG, de Oliveira LM. Effects of early postnatal malnutrition and chlordiazepoxide on experimental aversive situations. Physiol Behav 1992;51:1195–9.

Almeida SS, Tonkiss J, Galler JR. Malnutrition and reactivity to drugs acting in the central nervous system. Neurosci Biobehav Rev 1996; 20:389–402.

Ator NA, Griffiths RR. Discriminative stimulus effects of atypical anxiolytics in baboons and rats. J Pharmacol Exp Ther 1986;237: 393–403.

Ator NA, Griffiths RR. Differential generalization to pentobarbital in rats trained to discriminate lorazepam, chlordiazepoxide, diazepam, or triazolam. Psychopharmacology 1989;98:20–30.

Bailey CDC, Brein JF, Reynolds JN. Altered $GABA_A$-Benzodiazepine receptor number and pharmacology in the adult guinea pig cerebral cortex after chronic prenatal ethanol exposure. Alcohol: Clin Exp Res 1999; 23:1816–24.

Brioni JD, Orsingher OA. Operant behavior and reactivity to the anticonflict effect of diazepam in perinatally undernourished rats. Physiol Behav 1988;44:193–8.

Brioni JD, Cordoba NE, Orsingher OA. Decreased reactivity to the anticonflict effect of diazepam in perinatally undernourished rats. Behav Brain Res 1989;34:159–62.

Bronson M, Chen HC. Time course of discriminative stimulus effects of bretazenil and chlordiazepoxide in rats. Eur J Pharmacol 1996;305: 7–12.

Butler PD, Susser ES, Brown AS, Kaufmann CA, Gorman MD. Prenatal nutritional deprivation as a risk factor in schizophrenia: preclinical evidence. Neuropsychopharmacology 1994;11:227–35.

Costa ET, Savage DD, Valenzuela CF. A review of the effects of prenatal or early postnatal ethanol exposure on brain ligand-gated ion channels. Alcohol: Clin Exp Res 2000;24:706–15.

De Vry J, Slangen JL. Differential interaction between chlordiazepoxide, pentobarbital and benzodiazepine antagonists Ro 15-1788 and CGS 8216 in a drug discrimination procedure. Pharmacol, Biochem Behav 1986;24:999–1005.

Galler JR, Shumsky JS, Morgane PJ. Malnutrition and brain development. In: Walker WA, Watkins J, editors. Nutrition in pediatrics. Neuilly-sur-Seine, France: BC Decker, 1996. p. 196–212.

Little JZ, Teyler TJ. $GABA_a$ receptor-mediated field potentials are enhanced in area CA1 following prenatal cocaine exposure. Dev Brain Res 1998;110:115–9.

McNamara RK, Skelton RW. Effects of intercranial infusions of chlordiazepoxide on spatial learning in the Morris water maze: I. Neuroanatomical specificity. Behav Brain Res 1993;59:175–91.

Morgane PJ, Austin-LaFrance RJ, Bronzino JD, Tonkiss J, Diaz-Cintra S, Cintra L, Galler JR. Prenatal malnutrition and development of the brain. Neurosci Biobehav Rev 1993;17:91–128.

Nielsen EB, Valentine JD, Holohean AM, Appel JB. Benzodiazepine receptor mediated discriminative cues: effects of GABA-ergic drugs and inverse agonists. Life Sci 1983;33:2213–20.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H. Benzodiazepine actions mediated by specific γ-aminobutyric acid A receptor subtypes. Nature 1999;401: 796–800.

Sanger DJ. Further investigation of the stimulus properties of chlordiazepoxide and zolpidem. Agonism and antagonism by two novel benzodiazepines. Psychopharmacology 1987;93:365–8.

Sanger DJ, Benavides J. Discriminative stimulus effects of ω (BZ) receptor ligands: correlation with in vivo inhibition of [^{3}H]-flumazenil binding in different regions of the rat central nervous system. Psychopharmacology 1993;111:315–22.

Schechter MD, Signs SA, Boja JW. Stability of the stimulus properties of drugs over time. Pharmacol, Biochem Behav 1989;32:361–4.

Sieghart W. Unraveling the function of the $GABA_A$ receptor subtypes. TiPS 2000;21:411–3.

Teissere JA, Czajkowski C. A β-strand in the γ_2 subunit lines the benzodiazepine binding site of the $GABA_A$ receptor: structural rearrangements detected during channel gating. J Neurosci 2001;21:4977–86.

Tomie A, Shultz PL, Spicer MS, Peoples LL. Drug discrimination training with low doses: maintenance of discriminative control. Pharmacol, Biochem Behav 1995;50:115–9.

Tonkiss J, Galler JR, Morgane PJ, Bronzino JD, Austin-LaFrance RJ. Prenatal protein malnutrition and postnatal brain function. Ann NY Acad Sci 1993;678:215–27.

Tonkiss J, Shultz PL, Shumsky JS, Fiacco TA, Vincitore M, Rosene DL, Galler JR. Chlordiazepoxide-induced spatial learning deficits: dose-dependent differences following prenatal malnutrition. Pharmacol, Biochem Behav 2000a;65:105–16.

Tonkiss J, Trzcinska M, Shultz P, Vincitore M, Galler JR. Prenatally protein-malnourished rats are less sensitive to the amnestic effects of medial septal infusions of chlordiazepoxide. Behav Pharmacol 2000b; 11:437–46.

Young R, Glennon RA. Stimulus properties of benzodiazepines: correlations with binding affinities, therapeutic potency, and structure activity relationships (SAR). Psychopharmacology 1987;93:529–33.