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A Liquid Diet Model of Chlordiazepoxide Dependence in Mice[1]

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CHAN, A. W. K., F. W. LEONG, D. L. SCHANLEY, M. C. LANGAN AND M. L. PENETRANTE. *A liquid diet model of chlordiazepoxide dependence in mice.* PHARMACOL BIOCHEM BEHAV **34**(4) 839–845, 1989.—Mice fed chronically (3 to 4 weeks) a liquid diet containing chlordiazepoxide (CDP) became physically dependent on the drug as demonstrated by the occurrence of withdrawal signs precipitated by injection of the benzodiazepine antagonist Ro15-1788 (5 to 25 mg/kg) or by omitting CDP from the diet (spontaneous withdrawal). Very low blood concentrations of CDP, but medium to high levels of the active metabolites N-desmethyl CDP and demoxepam were found during the period of CDP administration. The Ro15-1788-induced withdrawal signs appeared within 1 min after the injection of the antagonist and lasted for at least 10 min. Quantifiable withdrawal signs included tail lift, tremor, impaired movement and handling-induced seizures. Mice undergoing spontaneous withdrawal had milder withdrawal signs such as weight loss, loss in appetite and suppression of runway and head-dipping activities on day 1 or day 2 of withdrawal. These signs were also present in Ro15-1788-induced withdrawal. A long-lasting rebound increase in runway and head-dipping activities occurred several days after CDP withdrawal.

Chlordiazepoxide dependence Benzodiazepine Withdrawal signs Ro15-1788 Behavioral tests

THE benzodiazepines (BZD) are widely used in the treatment of anxiety disorders (29) and alcohol withdrawal symptoms (25). Some clinicians (18,39) have advocated not using BZD in the long-term treatment of alcoholics beyond detoxification, citing their ineffectiveness, possible insidious long-term side effects, possible arousal or reinforcement of the "craving" for alcohol and potential danger of addiction. Another important consideration is whether the therapeutic use of BZD is counterproductive in alcoholics who are also abusers of BZD. Although there are anecdotal reports which suggest that alcoholics also use and may abuse BZD (2), information has only become available recently about the abuse of BZD in alcoholics. Several studies indicate that 5 to 23% of alcoholics are also abusers of BZD (1, 9, 16, 28, 31). Wiseman and Spencer-Peet (36) found that 76% of their alcoholic patients also took other drugs during the two weeks before alcoholism treatment. Of these, 86% had taken alcohol with their drugs; the most commonly taken drugs were BZD (41%). Other indirect evidence also suggests that the combined use of alcohol and BZD is quite common in alcoholics and the general population; this comes from data on drug use in traffic accident victims and drug overdose patients (2,24). Therefore, it is highly desirable and clinically relevant to learn about the long-term effects of the combined use of alcohol and BZD, as well as how these two drugs interact during their combined chronic intake. Very little is known about these important topics. Since ethical and humane considerations dictate against well-controlled studies on combined drug dependence in man, animal models have to be substituted.

As a prelude to developing a mouse model of combined ethanol/CDP dependence, we needed to develop a model of CDP dependence that would be compatible with the liquid diet model of ethanol dependence which involved the use of ethanol-preferring C57BL/6J mice (6, 7, 30). We have demonstrated previously (6,7) that the incorporation of CDP in a liquid diet containing ethanol may be a feasible approach to developing a model of ethanol/CDP dependence. However, the concentrations of CDP used in these studies were too low to elicit CDP dependence. This paper reports our investigation on the chronic feeding of a liquid diet containing CDP to induce CDP dependence in mice. Other animal models of BZD dependence have been developed in rats (20, 23, 32, 35, 38), mice (12,35), cats (8), dogs (21), monkeys (37) and baboons (17,19).

METHOD

Materials

CDP hydrochloride, diazepam, N-desmethyl CDP, demoxepam, and Ro15-1788 were kindly provided by Hoffmann-La Roche, Inc. (Nutley, NJ). A chocolate-flavored Sustacal liquid diet was purchased from Mead Johnson & Co. (Evansville, IN).

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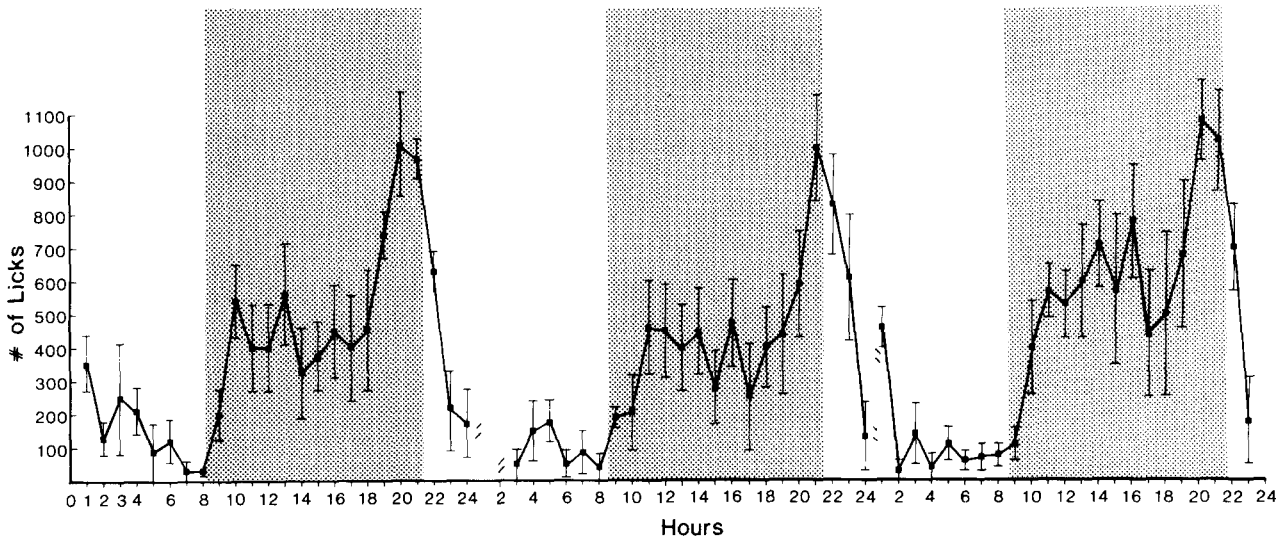

FIG. 1. Drinking pattern of CDP diet at the beginning, middle and late phases of the diet administration period. Each value (± S.E.; N = 8) represents the mean cumulative number of licks for 1 hr, except the 24th hour reading which reflects drinking activities for only a half hour. This was because the lickometer was turned off for 30 min to allow for recording volumes of consumption and administering fresh diets. The shaded area indicates the hours when lights in the room were turned off. Zero hour (approximately 8:30 a.m.) was when fresh diet was administered and the lickometer was switched on.

A vitamin diet fortification mixture (ICN Biochemicals, Cleveland, OH) was used with the liquid diet. Sucrose was purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Male C57BL/6J mice (8–9 weeks old) were purchased from the Jackson Laboratories (Bar Harbor, ME). They were housed singly in plastic cages in a controlled-environment room (21–22°) on an 11/13 hr light/dark cycle, and received Teklad mouse diet (Teklad Mills, Winfield, IA) and tap water ad lib for at least one week before the beginning of an experiment.

Administration of CDP Diet

Mice were divided into groups (N = 10 to 12 in each group) such that their mean body weights were similar. The number of groups depended on the types of experiments to be performed. Basically, for each group of mice which was fed ad lib the liquid diet containing CDP (see below) there was a control group which was pair-fed the same liquid diet except that CDP was omitted (control diet). The control diet was composed of the following: for each liter, Sustacal (739 ml), sucrose (106.6 g), vitamin mixture (3 g), and tap water (192 ml). Routinely, a two-day supply was prepared and stored in a refrigerator. To prepare the CDP diet, the control diet minus the required volume of water was used; the volume of water which was omitted from the diet was used to dissolve the CDP hydrochloride (see below for concentrations) and the CDP solution was then blended briefly with the modified control diet.

We have experimented with different combinations of starting doses of CDP, daily CDP increments and duration of diet administration. The following procedure is of shorter duration and involves higher concentrations of CDP in the diet than the one reported in a preliminary report (3). Mice were fed ad lib the control diet as the only source of food and fluid for 3 days to allow them to get accustomed to the liquid diet. Thereafter, CDP (0.6 mg/ml) was incorporated in the diet and maintained for 3 days, followed by an increase to 1 mg/ml for another 3 days. From then on the CDP concentration was increased daily by 0.1 mg/ml for 18–25 days. Initially, when the daily intake volume exceeded 13 ml, we used 50 ml plastic graduated centrifuge tubes for the administration of diet. In the later stage, 15 ml graduated tubes were used. Tubes containing the CDP diet were wrapped with aluminum foil because CDP is photosensitive. The volume of daily diet intake was recorded, and the mice were weighed twice each week. From the known CDP concentration in the diet, the body weight of each mouse, and the volume of diet intake, the daily CDP intake (mg/kg) could be calculated.

Diet Drinking Pattern

A lickometer device was used to determine the hourly drinking pattern in some mice during chronic CDP diet administration. The apparatus has been described previously (4).

Withdrawal Reactions

On the day that the CDP diet was to be withdrawn, the control diet was given ad lib to the CDP-treated mice. Control mice were still pair-fed the control diet, but one subgroup was fed the diet ad lib so that comparisons could be made with CDP-dependent mice about daily diet intake during withdrawal. The diet administration during CDP withdrawal lasted 1 to 3 days, depending on when the CDP-dependent mice began to consume excessive amounts (over 15 ml) of the diet. When this happened all the mice were given food pellets and water ad lib. This was to prevent the mice from getting obese as a result of consuming large amounts of the control diet. Two kinds of withdrawal reactions were monitored, namely, Ro15-1788-induced withdrawal and spontaneous withdrawal.

Ro15-1788-induced withdrawal. Within an hour after the removal of CDP diet, mice were injected intraperitoneally (IP) with Ro15-1788 (5 to 25 mg/kg). A fine suspension of this drug was prepared in distilled water by addition of Tween-80 (2 drops per 10 ml) and vigorous mixing. Because of the short time course of the precipitated withdrawal symptoms, only several withdrawal signs

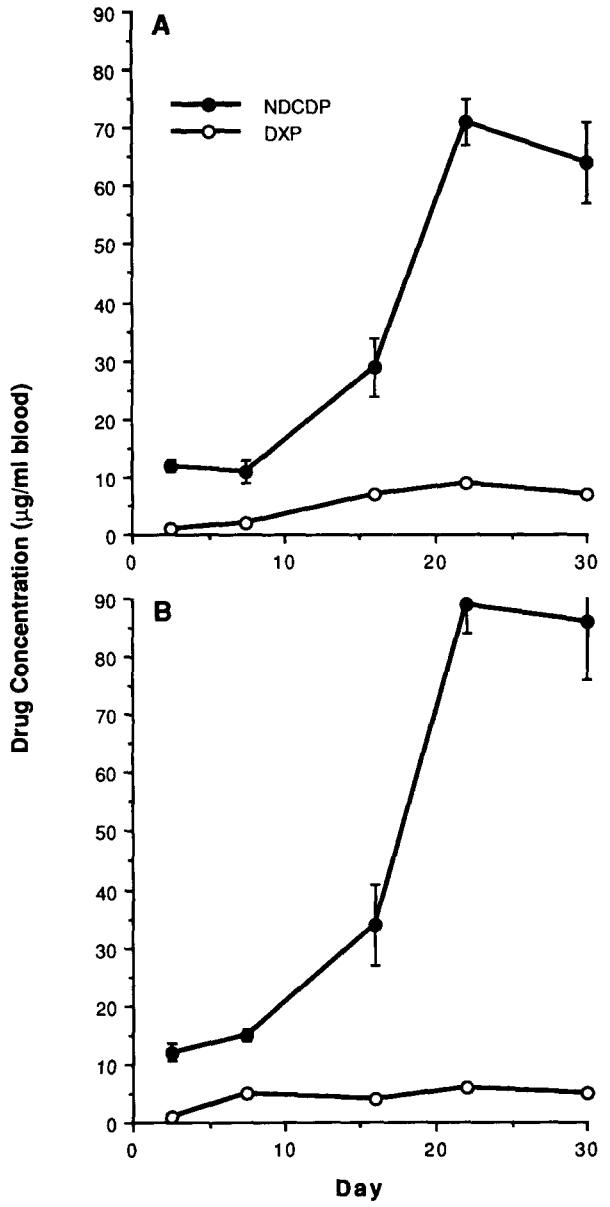

FIG. 2. Blood levels of N-desmethyl CDP (NDCDP) and demoxepam (DXP) on selected days during CDP diet administration. Blood samples were taken at 9:30 a.m. (panel A) and 9:30 p.m. (panel B) on the days shown. Mean values (±S.E. for NDCDP only) for N = 11 are plotted. Day 1 was the first day that CDP was incorporated in the liquid diet. The respective daily CDP intake for the days shown were 326±20, 631±65, 714±26, 914±66 and 1007±75 mg/kg.

were scored according to the following scoring system: (a) tremor, 0 = none, 1 = fine body temor, 2 = coarse tremor with mildly impaired locomotion, 3 = marked coarse tremor with marked impairment of locomotion, 4 = severe coarse tremor and falls during locomotion; (b) tail lift, 0 = flattened to ground, 1 = horizontal, 2 = 45° lift, 3 = 90° lift, 4 = retrograde over back. The scoring for these two signs was modified from the system reported by Gallaher *et al.* (12); (c) handling-induced seizures (14); mouse was picked up by the tail, 0 = no seizure when turned 180°, 1 = seizure occurred when turned 180°, 2 = seizure occurred when gently "tickled" near the lower abdominal area, 3 = seizure occurred when mouse was picked up, 4 = spontaneous seizures in home cage; (d) impairment in locomotion, 0 = normal movement with some rearing, 1 = slowed movement with diminished rearing, 2 = slow and deliberate movement with absence of rearing, 3 = very slow movement, no rearing, 4 = turning very slowly in circle with occasional backward movement. These signs were scored at 1 min intervals for 10 min following the injection of Ro15-1788. Other quantitative assessment of withdrawal symptoms included changes in body weights (determined daily for several days after CDP diet withdrawal), daily diet intake and behavioral tests (see below).

Spontaneous withdrawal. After removal of the CDP diet, the mice were fed ad lib the control diet. Control mice continued to be pair-fed the control diet. The scoring system for Ro15-1788-induced withdrawal could not be used because such withdrawal signs were infrequent, hard to detect or virtually absent in spontaneous withdrawal. Instead, the following were monitored: changes in body weights and volume of diet intake, as well as behavioral tests as described below.

Blood Levels of CDP

Tail blood samples (50 μl) were collected at weekly intervals from the mice which were fed the CDP diet. Because of the differences in drinking patterns between day and night hours, one sample was taken between 8:30–9:30 a.m. and another one between 8:30–9:30 p.m. The blood samples were extracted and analyzed for CDP, N-desmethyl CDP (NDCDP) and demoxepam (DXP) by high pressure liquid chromatography according to published procedures (7,15).

Behavioral Tests

The runway and head-dipping tests were used to assess the performance of mice undergoing CDP withdrawal. Separate batches of mice were used for each test. No drug injections were given to the mice before these tests. The apparatus and testing procedure for the runway test have been described previously (5). In this test, the number of complete runs from one end of the runway to the other during a 5-min test period was determined; the time elapsed before the mouse completed its first run was also recorded. The head-dipping apparatus was similar to that described by File (10), except that there was no automatic monitoring of locomotor activity. The number of head-dips and the total time that the mouse spent on head-dipping during a 7-min test period were recorded automatically. We also determined the time elapsed before the mouse made its first dip.

RESULTS

Diet Consumption and Blood BZD Levels

Figure 1 shows the typical 24-hr diet consumption (licking) patterns on three separate days (beginning, middle, and late phases of the diet administration period). The data indicate that increasing the concentration of CDP in the diet did not change the drinking pattern. In general, the mice consumed more diet during the hours of darkness. The respective daily CDP intake (computed from volume of intake, concentration of CDP in diet and body weight) for the days shown in Fig. 1 were 326±20, 747±117, and 1205±75 mg/kg. Body weight (g) at the beginning of the diet period, peak body weight and weight on the last day of CDP diet administration were: 24.57, 26.85 (day 22) and 26.04 (day 33), respectively.

Figure 2 depicts blood levels of NDCDP and DXP during the period of CDP diet administration. The concentration of CDP was

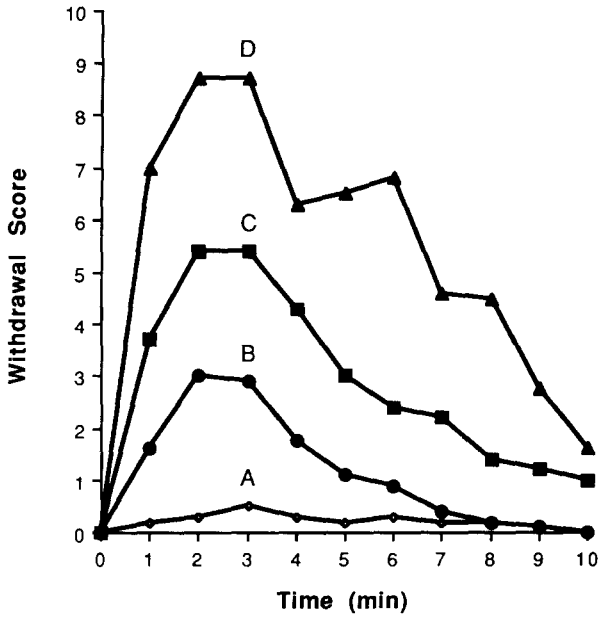

FIG. 3. Withdrawal scores during Ro15-1788-induced withdrawal. Ro15-1788 (B, 5 mg/kg; C, 15 mg/kg; D, 25 mg/kg) or vehicle (A) was injected at time zero. Mean values for the combined withdrawal signs (see the Method section) in each group (N = 9 to 11) are plotted.

either undetectable or below 1.5 μg/ml. In general, blood samples taken in the evening (panel B) had slightly higher levels of NDCDP than those taken in the morning (panel A). However, DXP concentrations were comparable for the two time periods. Despite daily increases of CDP concentrations in the liquid diet, blood levels of NDCDP on days 22 and 30 were comparable. This was probably caused by a combination of a slight decrease in daily diet intake as the CDP concentrations increased and an increase in the metabolism of the metabolites of CDP (Chan, unpublished results).

Ro15-1788-Induced and Spontaneous Withdrawal

Quantifiable withdrawal reactions occurred within one minute after the injection of Ro15-1788 (Fig. 3). There were dose-related increases of withdrawal intensity between 5 and 25 mg/kg doses of Ro15-1788. The more prominent signs were tail lift and slow movement, while the score for handling-induced seizures was usually 1. Thus, spontaneous seizures were not observed except in one experiment in which the CDP diet administration (with more gradual increases of CDP concentrations in the diet) lasted over 80 days. In this instance, about 20–30% of the mice had sporadic spontaneous seizures on day 3 and day 4 after CDP withdrawal. As Fig. 3 indicates, most of the withdrawal signs had subsided by 10 min. If another dose of Ro15-1788 was injected 1 or 4 hr after the first injection, withdrawal signs reappeared with intensities and time course comparable to those shown in Fig. 3. However, if the BZD antagonist was reinjected 24 hr after the first injection, no quantifiable withdrawal signs could be detected. Blood samples taken from mice at 24 hr after CDP withdrawal did not have detectable levels of CDP, NDCDP and DXP. Therefore, it appears that Ro15-1788 could only induce withdrawal reactions in mice which still had residual levels of CDP, NDCDP, or DXP in their bodies.

In contrast to the Ro15-1788-induced withdrawal, mice undergoing spontaneous withdrawal did not show well-defined and easily quantified withdrawal signs as those shown in Fig. 3. Nevertheless, gross symptoms such as weight loss and loss of appetite could be quantified. Table 1 compares body weight changes in mice which had Ro15-1788-induced or spontaneous withdrawal. Since there were no significant differences in weight changes among mice which were injected with different doses of Ro15-1788, only the results for mice injected with 25 mg/kg Ro15-1788 were included in the table. Mice which had spontaneous withdrawal lost more weight after day 1 (Δ1) and day 2 (Δ2) than those which had Ro15-1788-induced withdrawal, but the difference was only significant for Δ2, $F(1,36) = 6.34$, $p = 0.01$. In fact, after the second day of withdrawal, mice which were injected with Ro15-1788 on day 1 had recovered from the weight loss, while the mice in spontaneous withdrawal still showed a mean weight loss of 0.67 g. The decrease in body weight in Ro15-1788-induced or spontaneous withdrawal appeared to be part of the withdrawal phenomena rather than solely a result of reduced diet intake on day 1 of CDP withdrawal. This is because the weight loss in pair-fed control mice was much less than that seen in mice undergoing either type of CDP withdrawal (Table 1). Compared to the intake of CDP diet on the last day of CDP administration, the mean volume of control diet consumed by mice on day 1 of spontaneous withdrawal was decreased by 53%, while that consumed by mice on day 1 of Ro15-1788-induced withdrawal was decreased by 22%. By day 2 of withdrawal, mice undergoing either type of withdrawal regained their appetites and the volume of control diet consumed was 30 to 45% more than that of CDP diet consumed on the day before withdrawal.

TABLE 1

CHANGES IN BODY WEIGHT DURING CDP WITHDRAWAL

Ro15-1788 Dose (mg/kg)	Weight Changes (g)* Δ1	Δ2	Δ3
	CDP-Dependent Mice		
0† (N=20)	−2.35 ± 0.20	−0.67 ± 0.16	−0.12 ± 0.08
25 (N=18)	−1.90 ± 0.23	0.21 ± 0.32	0.36 ± 0.29
	Pair-Fed Control Mice		
0† (N=22)	−0.14 ± 0.05	1.28 ± 0.35	0.81 ± 0.21
25 (N=20)	−0.32 ± 0.12	0.81 ± 0.23	0.61 ± 0.32

*Mice were weighed on the morning of CDP diet withdrawal (initial weight) and several mornings thereafter. Each weight change (Δ) represents the difference between the weight recorded at the end of the day specified (e.g., 1 = Day 1 and so on) and the initial weight. Values are means ± S.E.

†Spontaneous withdrawal.

Behavioral Tests

Runway and head-dipping activities after CDP withdrawal are depicted in Fig. 4 and Fig. 5, respectively. Only data from mice which underwent spontaneous withdrawal are shown because similar results were obtained in mice which had Ro15-1788-induced withdrawal. Prior to CDP diet administration, the different groups of mice did not differ significantly in the parameters measured. Moreover, our preliminary experiments (data not shown) also indicate that the behaviors of pair-fed control mice were comparable to those which were fed ad lib with food pellets and water for the same duration as that for liquid diet

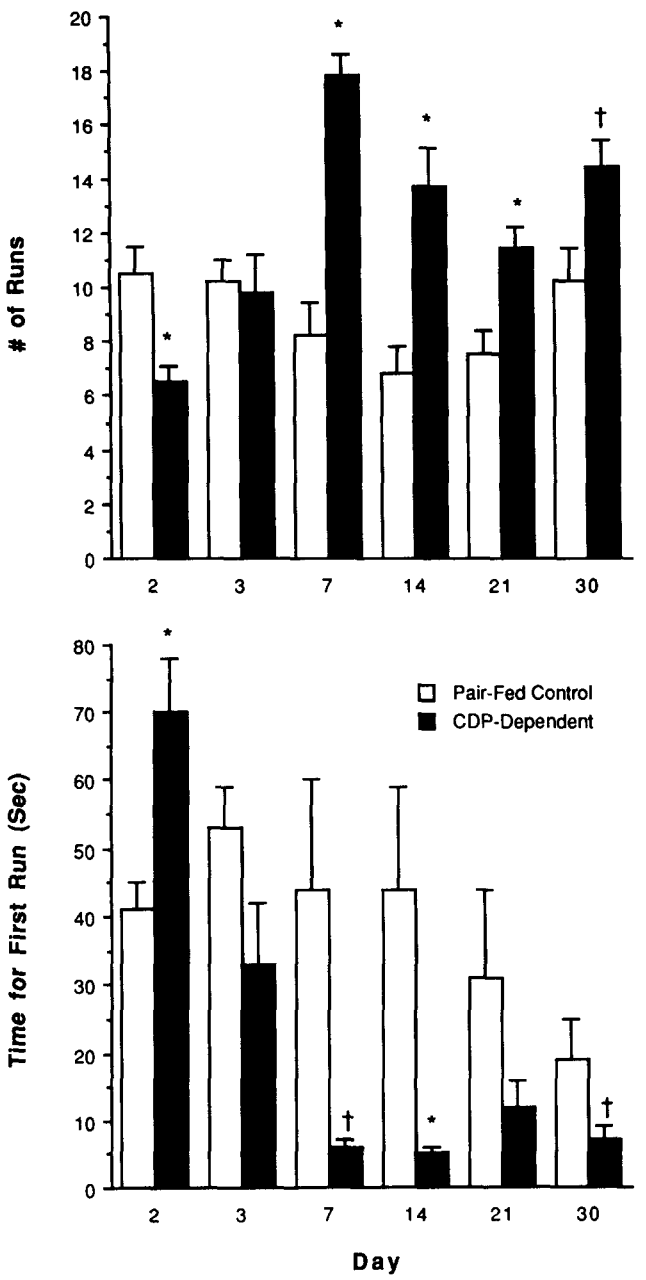

FIG. 4. Runway activity (no drug injection) after spontaneous CDP withdrawal. Day 1 was when the CDP diet was withdrawn. Values are means ± S.E. N = 17 for CDP-dependent mice and N = 12 for pair-fed control mice. *$p<0.005$, †$p<0.05$.

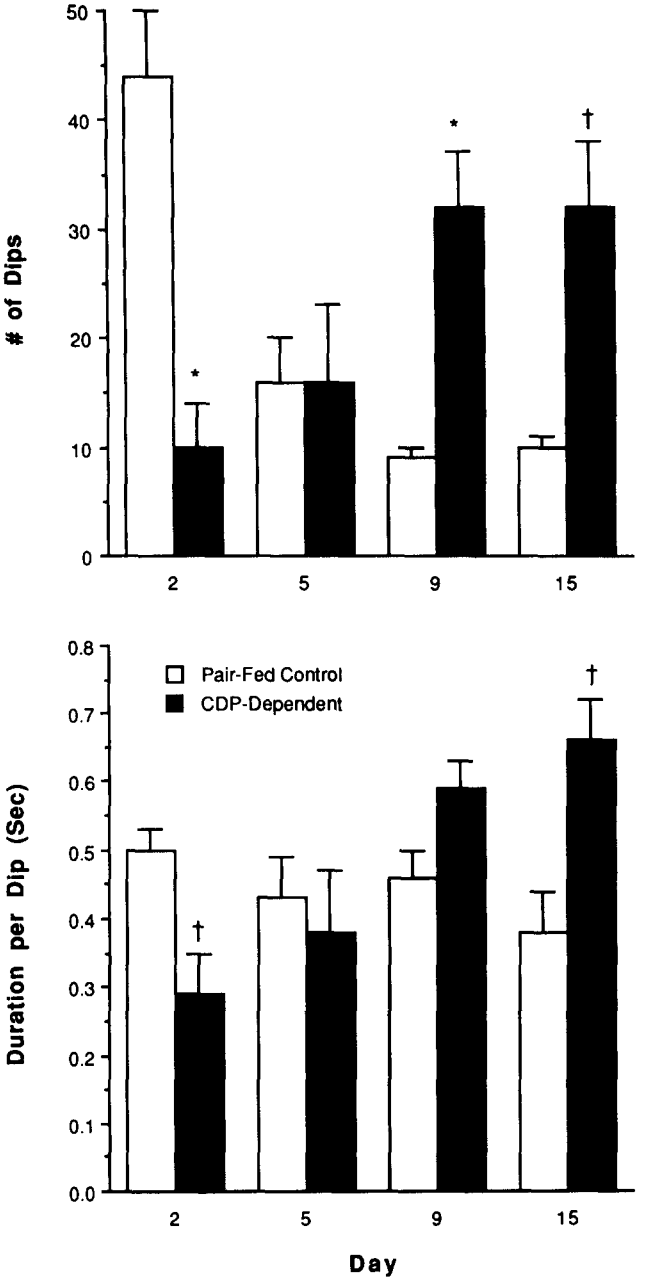

FIG. 5. Head-dipping activity (no drug injection) after spontaneous CDP withdrawal. Day 1 was when the CDP diet was withdrawn. Values are means ± S.E. N = 11 to 12 in each group. *$p<0.001$, †$p<0.03$.

administration. Therefore, the control diet treatment per se did not alter the behaviors of these mice. On day 2 of withdrawal, CDP-dependent mice had decreased runway activity, making significantly fewer complete runs, $F(1,27) = 18.6$, $p<0.005$, and taking longer, $F(1,27) = 24.1$, $p<0.005$, to make the first run than pair-fed control mice (Fig. 4). In contrast, from day 7 onwards, CDP-dependent mice made significantly more runs and had shorter time for first run than pair-fed control mice [e.g., on day 7, $F(1,27) = 41.9$, $p<0.001$, for number of runs, and $F(1,27) = 7.3$, $p = 0.001$, for first run time]. These behaviors were highly reproducible. In a similar experiment, we found that there was also a suppression of runway activity on day 1 of CDP withdrawal and that the rebound increase in runway activity was detectable by day 5 of withdrawal. Although the data shown in Fig. 4 only include testing up to day 30 of withdrawal, results from another experiment indicate that the increase in runway activity was still detectable 4 months after CDP withdrawal.

In general, head-dipping activity (Fig. 5) followed a trend similar to that of runway activity, with CDP-dependent mice making significantly fewer dips, $F(1,21) = 22.3$, $p<0.001$, and having a shorter duration per dip, $F(1,21) = 6.7$, $p<0.05$, than

pair-fed control mice on day 2 of withdrawal. By day 9 the CDP-dependent mice showed an increase in head-dipping activity. Results of a separate experiment indicate that the increase in head-dipping activity was detectable on day 40 after CDP withdrawal.

DISCUSSION

The liquid diet method provides a convenient way of inducing CDP dependence in mice. Results of the drug assays indicate that blood levels of CDP were very low and the mice were exposed more to high levels of NDCDP, and to appreciable levels of DXP. Therefore, it can be argued that NDCDP and DXP contributed significantly to the development of "CDP" dependence. However, since we did not measure brain CDP levels, we cannot rule out the possibility that the chronic exposure to low levels of CDP in the mouse's brain might be sufficient to induce dependency on the drug. A similar finding was reported by Gallaher *et al.* (12) in their mouse model of diazepam (DZP) dependence which was elicited by feeding mice food pellets containing the drug. These investigators found that circulating levels of DZP were very low, and the mice were primarily exposed to the two active metabolites, namely, nordiazepam (NDZP) and oxazepam. Although the dependence liabilities of the metabolites of CDP or DZP have not been compared thoroughly with their respective parent drugs, McNicholas *et al.* (22) reported that the abstinence syndrome observed after abrupt discontinuation of NDZP in dogs was similar to the DZP withdrawal syndrome, but differed in several aspects. One important difference was that the withdrawal scale was greater in NDZP-dependent dogs than in dogs dependent on DZP. These investigators suggested that physical dependence on DZP was caused by the accumulation and actions of NDZP. Similarly, our results indicate that in mice physical dependence on CDP is caused primarily by the accumulation of NDCDP and, to a lesser extent, of DXP. The relative dependence liabilities of CDP and NDCDP in humans are difficult to determine because of practical and ethical constraints, but it is very likely that in these instances accumulation of NDCDP contributes importantly to the development of CDP dependence. Schmuss *et al.* (33) reported that in patients who were dependent on high doses of DZP, peak withdrawal appeared when the serum NDZP level dropped significantly.

Several studies have reported that in animals (cat, rat and baboon) which were dependent on BZD, the onset of Ro15-1788-induced withdrawal symptoms occurred within 5 to 20 min after injection of Ro15-1788 (13, 17, 20). Our results indicate that in mice Ro15-1788-induced withdrawal signs appeared within 1 min of Ro15-1788 injection. The rapid dissipation of these signs (Fig. 3) was probably due to the rapid metabolism of Ro15-1788 in mice (5). As expected, mice which had Ro15-1788-induced withdrawal also showed spontaneous withdrawal signs such as weight loss, loss of appetite and locomotor impairment on day 1 and day 2 of CDP withdrawal. In contrast, signs such as tail lift, tremor and handling-induced seizures, which were easily scored in Ro15-1788-induced withdrawal, could not be detected in sufficiently predictable or regular frequencies to allow for reliable quantifications during a designated time course. Therefore, the mice in the present study had a much less severe spontaneous withdrawal compared to that reported for rats which were given chronic oral intubation of hypnotic doses of CDP for 5 weeks (32). In the rat study, the latency to onset of withdrawal ranged from 2 to 5 days, and withdrawal signs such as tremors, piloerection, arched back, etc., were protracted, peaking in 8 days and disappearing by 14 days postwithdrawal. The following factors may contribute to the differences between our study and that of Ryan and Boisse (32): method and dosage of drug administration as well as species differences in sensitivity to CDP and rate of metabolism of CDP.

Mice which had either Ro15-1788-induced or spontaneous withdrawal exhibited impairment of runway and head-dipping activities on the first two days of withdrawal. By day 3 of withdrawal the behavioral activities returned to normal but there were long-lasting rebound increases after that (Figs. 4 and 5). There has been no previous investigation of the long-term effects of chronic CDP treatment in mice. One study reported that 4 and 10 weeks after 12 days of 5 mg/kg CDP administration in rats, the animals had an increased resistance to extinction and punishment and decreased susceptibility to seizures, respectively. In humans, rebound anxiety and insomnia have been described after chronic BZD use (11, 26, 27). The rebound phenomenon is probably a reflection of drug-induced adaptive responses. The liquid diet method provides a convenient model to study the long-term effects of BZD administration in mice, e.g., tolerance to BZD and cross-tolerance to other drugs such as ethanol.

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