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Does Chronic Ethanol Intake Confer Full Cross-Tolerance to Chlordiazepoxide?[1]

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CHAN, A. W. K., M. C. LANGAN, F. W. LEONG, D. L. SCHANLEY AND M. L. PENETRANTE. *Does chronic ethanol intake confer full cross-tolerance to chlordiazepoxide?* PHARMACOL BIOCHEM BEHAV 30(2) 385–389, 1988.—Four behavioral tests, namely, hypothermia, horizontal dowel, runway and head-dipping, were used to assess tolerance to ethanol and cross-tolerance to chlordiazepoxide (CDP) in mice chronically treated with an ethanol diet for 15 days. Mice were tested on day 3 of ethanol withdrawal, with some being retested on day 8. In terms of hypothermia and the horizontal dowel test, ethanol tolerance conferred full cross-tolerance to CDP, but the conclusion based on results of the latter test may be equivocal. Partial cross-tolerance to CDP was observed in the runway test, while no cross-tolerance to CDP was detected in the head-dipping test. For these latter two tests ethanol tolerance was present in the mice. Thus, the degree of equivalence between tolerance to ethanol and cross-tolerance to CDP in ethanol-dependent mice varied with the behavioral tests to assess tolerance. Possible mechanisms are discussed.

Ethanol Chlordiazepoxide Ethanol tolerance Cross-tolerance to chlordiazepoxide Behavioral tests

THERE is very limited information available for the relationship between ethanol tolerance and cross-tolerance to benzodiazepines (BZD) after chronic intake of ethanol in animals or men. Although cross-tolerance between ethanol and BZD is often assumed or stated as well-documented [12, 16, 23], there have been only a few reports documenting such a phenomenon [5, 15, 18, 22]. Rosenberg *et al.* [22] found that rats treated chronically with flurazepam in their drinking water developed tolerance to the drug and they were also cross-tolerant to ethanol. Rats fed chronically a liquid diet containing ethanol showed an attenuated response to an anesthetic dose of diazepam [18]. Le *et al.* [15] reported that treatment of rats with chlordiazepoxide (CDP) conferred full cross-tolerance to ethanol and pentobarbital; however, prior treatment with ethanol only conferred partial cross-tolerance to CDP. We have shown previously [5] that an acute dose of ethanol elicited a rapid tolerance to ethanol and cross-tolerance to CDP in mice; however, an acute dose of CDP did not induce a rapid cross-tolerance to ethanol. Therefore, there is a need to investigate whether the extent of tolerance to ethanol in mice chronically exposed to ethanol is fully equivalent to that of cross-tolerance to CDP.

METHOD

Animals

Male C57BL/6J mice (8 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°C) 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. All behavioral tests were performed in the same room in which the mice were housed.

Materials

CDP-hydrochloride was kindly provided by Hoffmann-LaRoche, Inc. (Nutley, NJ). A chocolate-flavored Sustacal liquid diet was purchased from Mead Johnson & Co. (Evansville, IN). A vitamin diet fortification mixture (ICN Biochemicals, Cleveland, OH) was used with the liquid diet. Sucrose and Ethanol Kit were purchased from Sigma Chemical Co. (St. Louis, MO).

Induction of Ethanol Dependence

Mice were fed ad lib a liquid diet containing ethanol according to previously published procedure [4]. Briefly, the ethanol concentration in the diet was 3.5% (v/v) for the first 6 days; it was then increased by 1.5% every 3 days until the final concentration of 8% was maintained for 3 days. Thus, the total diet period was 15 days. The number of groups of mice depended on the tests to be performed after ethanol withdrawal, with each group having 9–12 mice. Control mice were pair-fed an isocaloric diet containing sucrose as a substitute for ethanol.

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TABLE 1

PROTOCOLS FOR DRUG INJECTIONS AND BEHAVIORAL TESTING

Test	ETOH Dose (g/kg)	CDP Dose (mg/kg)	Time of Test*
Hypothermia	2 or 3	60 or 90	0.5, 1, 2, 3, 4 hr†
Runway	2.25	30	15 min
Head-Dipping	2.5	40	15 min
Horizontal Dowel	1.75	160	20 sec

*Time after injection that the test was begun.

†Include rectal temperature determinations before drug injection (0 hr).

Tests for Ethanol Tolerance and Cross-Tolerance to CDP

On days 1 and 2 of ethanol withdrawal the ethanol-dependent mice were fed ad lib the control diet. Pair-feeding of the control mice was continued during this time. From then on all the mice received food pellets and water ad lib. The change from the liquid diet to solid food (after testing for tolerance or cross-tolerance on day 3) was necessary because the mice tended to consume large amounts of the liquid diet by day 2 of ethanol withdrawal and would have become obese if the feeding of control diet had been continued.

Mice were tested on day 3 of ethanol withdrawal; some were retested on day 8. The following tests were used: (a) *Drug-Induced Hypothermia* [6,21]: Rectal temperature was determined before and at several hourly intervals after saline, ethanol or CDP injection. (b) *Runway:* The apparatus has been described in detail in another publication [3]. 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. (c) *Head-Dipping* [7,9]: 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. (d) *Horizontal Dowel:* The apparatus was the same as that described by Goldstein and Zaechelein [11]. The mouse was gently restrained for 20 sec after ethanol or CDP injection and was then placed on the dowel. The time (seconds after injection) that the mouse fell off the dowel was recorded and the mouse was sacrificed by cervical dislocation immediately after falling. The whole brain was dissected and processed as described in Analytical Procedure. A fall-off time of 300 sec was assigned to the mouse if it did not fall off by 5 min after drug injection.

Table 1 summarizes drug doses and testing protocols. The selections of doses for CDP and ethanol were based on pilot data which indicated that the selected doses of ethanol and CDP for each test would yield nearly the same pharmacological effects in naive mice. For each behavioral test and drug dose, the difference between the response of ethanol-dependent mice and that of control mice was a measure of the magnitude (extent or degree) of ethanol tolerance (ethanol injection) or CDP cross-tolerance (CDP injection). If the magnitude of ethanol tolerance did not differ significantly from that of CDP cross-tolerance, full cross-tolerance to CDP was deemed to have developed. If the magnitude of CDP cross-tolerance was less than that of ethanol tolerance, partial cross-tolerance to CDP was deemed to have developed.

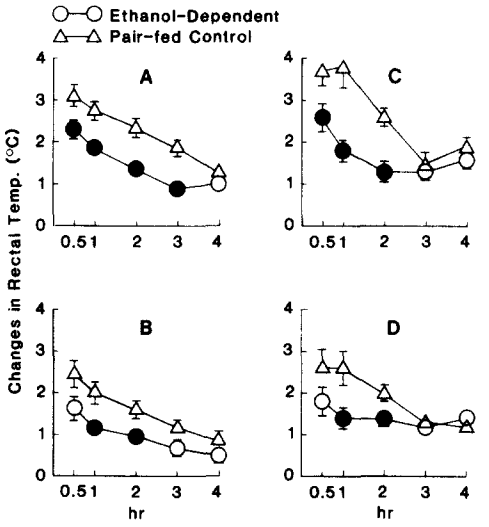

FIG. 1. Hypothermic responses in ethanol-dependent and control mice. Separate groups (N=11–13 each) of mice were injected either with ethanol (A, 3 g/kg; B, 2 g/kg) or CDP (C, 90 mg/kg; D, 60 mg/kg) on day 3 of ethanol withdrawal. Values are mean decreases (relative to zero hr values) ±S.E. Closed symbols indicate $p<0.01$ compared to controls.

Analytical Procedure

Brain ethanol levels were analyzed enzymatically using an Ethanol Kit according to published procedures [6,11]. The whole brain was homogenized in 9 volumes of cold 3.4% perchloric acid and the precipitate was removed by centrifugation. The supernatant was used for ethanol analysis. Brain CDP levels were determined by high pressure liquid chromatography according to previously published procedures [1,10].

Statistical Analysis

Significance of group differences was analyzed by ANOVA programs (Version 1.1, Human Systems Dynamics, Northridge, CA) with an Apple IIe computer. Where appropriate a 2×2 ANOVA was used to determine interaction effects.

RESULTS

Hypothermia

Figure 1 compares changes in rectal temperature in ethanol-dependent and control mice in response to challenge doses of either ethanol or CDP. Although our pilot experiments indicated that drug-naive mice not treated with the liquid diet showed comparable hypothermic responses to the respective doses of ethanol and CDP, the control mice tended to have slightly more CDP-induced hypothermia than that induced by the corresponding doses of ethanol. This was more evident with the CDP dose of 90 mg/kg from 0.5 to 2 hr (Fig. 1C). The ethanol-dependent mice developed significantly less hypothermia from the ethanol and CDP doses than the control mice did. These data clearly demonstrated tolerance to ethanol and cross-tolerance to CDP in the ethanol-dependent mice. Overall, there were no significant differences in the magnitudes (difference between ethanol-dependent and control mice) of ethanol tolerance and cross-tolerance to CDP, even though the magnitude of CDP cross-tolerance at 1 hr in Fig. 1C was apparently larger than the

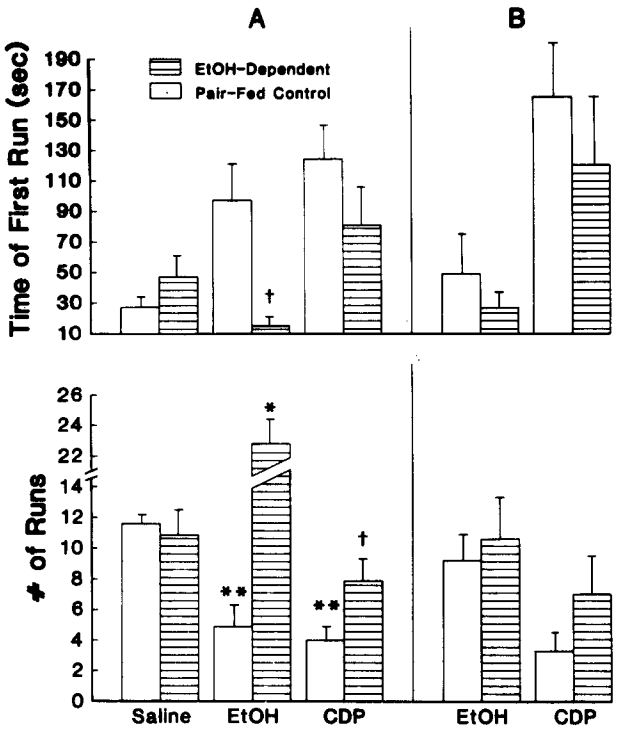

FIG. 2. Runway activities in ethanol-dependent and control mice. Panels A and B show results for testing on day 3 and day 8 of ethanol withdrawal, respectively. The ethanol dose was 2.25 g/kg and the CDP dose was 30 mg/kg. N=9 to 11 mice in each group. *$p<0.001$; †$p<0.01$. **$p<0.001$, compared to controls injected with saline.

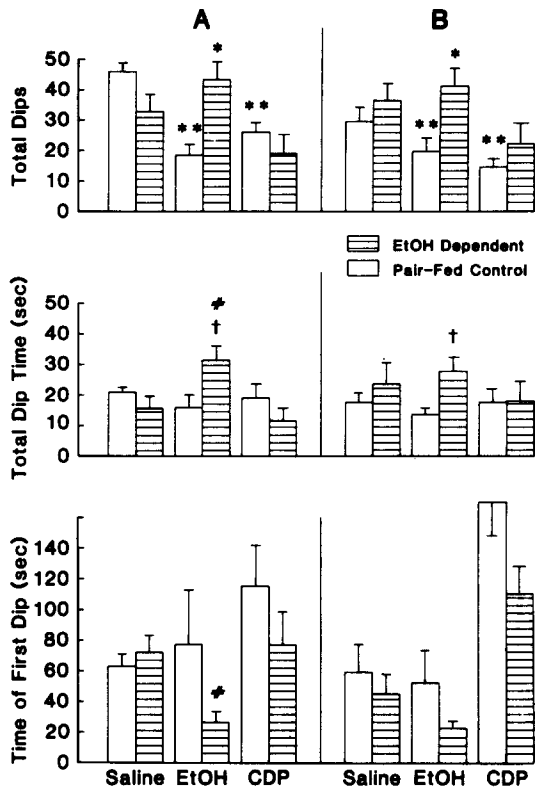

FIG. 3. Head-dipping activities in ethanol-dependent and control mice. Panels A and B depict results for days 3 and 8 of ethanol withdrawal, respectively. The ethanol dose was 2.5 g/kg and the CDP dose was 40 mg/kg. N=7 to 11 mice in each treatment group. *$p<0.005$, †$p<0.05$, compared to controls injected with ethanol. **$p<0.001$, compared to controls injected with saline. #$p<0.05$, compared to ethanol-dependent mice injected with saline.

magnitude of ethanol tolerance at 1 hr in Fig. 1A. Therefore, chronic ethanol diet treatment conferred full cross-tolerance to CDP, when drug tolerance was determined by this method.

Horizontal Dowel Test

The control mice injected with ethanol (1.75 g/kg) or CDP (160 mg/kg) showed comparable fall-off times (Table 2). These data suggest that the ethanol and CDP doses were equipotent. The ethanol-dependent mice showed increases (compared to controls) in ethanol and CDP fall-off times as well as increases in brain levels of ethanol and CDP at fall-off, indicating that these mice had developed tolerance to ethanol and cross-tolerance to CDP. The increase in ethanol fall-off time (194%) was much more than the increase in CDP fall-off time (61%), but the percent changes in brain drug levels between ethanol-dependent and control mice at the time the animals fell off were nearly the same for both drugs (ethanol, 33%; CDP, 35%). These latter results were taken as evidence that full cross-tolerance to CDP had developed in the ethanol-dependent mice, but the conclusion may be equivocal (see also the Discussion section).

Runway Test

The ethanol-dependent and control mice did not differ in the total number of runs and the time of first run when they were tested after saline injection (Fig. 2A). The control mice were almost equally affected by the ethanol (2.25 g/kg) or CDP (30 mg/kg) injection in that the number of runs was significantly decreased [for ethanol, $F(1,22)=22.7$, $p<0.001$; for CDP, $F(1,22)=63.4$, $p<0.001$] and they took significantly longer [for ethanol, $F(1,22)=8.8$, $p<0.01$; for CDP, $F(1,22)=19.1$, $p<0.001$] to make the first run, compared to control mice injected with saline. These data indicated that the doses of ethanol and CDP were equipotent. Compared to the controls, the ethanol-dependent mice that were injected with ethanol on day 3 of withdrawal showed a dramatic increase in the number of runs, $F(1,18)=71.4$, $p<0.001$, and a much shorter time to complete the first run, $F(1,18)=12.2$, $p=0.002$. The mean number of runs (22.8 ± 1.6) for the ethanol-dependent mice greatly surpassed that (mean value of about 11 runs) for mice injected with saline. Therefore, the dose of ethanol that caused inhibition in runway activities in control mice elicited an apparent stimulatory effect in the ethanol-dependent mice. The ethanol-dependent mice that were injected with CDP also showed a significant increase in the number of runs compared to control mice, $F(1,18)=7.0$, $p=0.015$, but the level of increase did not surpass the performance of saline-injected mice. There was no significant difference, $F(1,18)=1.5$, $p>0.2$, in the time of first run between the ethanol-dependent and control mice that were injected with CDP. Based on results of the number of runs, we concluded that the degree of tolerance to ethanol was quantitatively greater than the degree of cross-tolerance to CDP, when tolerance was measured on day 3 of ethanol withdrawal. No tolerance to ethanol or cross-tolerance to CDP was detectable on day 8 of ethanol withdrawal (Fig. 2B) by this test.

TABLE 2

Mouse Group	ETOH Fall-Off Time (sec)	Brain ETOH (mg/g)	CDP Fall-Off Time (sec)	Brain CDP (μg/g)
ETOH-dependent	250 ± 19*	2.03 ± 0.04*	151 ± 16*	28.6 ± 1.9*
Control	85 ± 8	1.53 ± 0.09	94 ± 10	21.1 ± 1.7

Separate groups (N=11–12 each) of mice were injected with ethanol (1.75 g/kg) or CDP (160 mg/kg).
Values are means ± S.E.
*$p<0.01$, compared to respective control.

Head-Dipping Test

The ethanol-dependent and control mice did not differ significantly in the three measures of head-dipping activity when they were tested after saline injection (Fig. 3A and 3B). The challenge doses of ethanol (2.5 g/kg) and CDP (40 mg/kg) produced comparable effects in the control mice, with only the total number of dips being significantly depressed by both drugs. Therefore, the number of dips was the only reliable measure of tolerance or cross-tolerance in this test. The total dip time was not significantly affected by ethanol or CDP injection in the control mice. The CDP-treated mice tended to have longer latency to first head-dip compared to saline-treated mice, especially on day 8 of withdrawal. This effect of CDP was more pronounced in control mice than in ethanol-dependent mice, but the differences were not significant on day 3 or day 8 of withdrawal. However, the difference in latency to first head-dip on day 8 of withdrawal between CDP-treated control mice and saline-treated control mice was significant, $F(1,19)=12.5$, $p<0.005$.

After an ethanol injection on day 3 of withdrawal, the ethanol-dependent mice made significantly more dips than the control mice, $F(1,19)=11.6$, $p<0.003$, so that the former group of mice behaved like saline-injected mice (Fig. 3A). The ethanol-dependent mice also spent more time in dipping $F(1,19)=6.5$, $p<0.05$; however, the time for first dip was not significantly different, $F(1,19)=1.8$, NS, from that in control mice because of large variations in the latter group. But compared with ethanol-dependent mice injected with saline, the ethanol-dependent mice injected with ethanol had a significantly shorter time of first dip, $F(1,17)=13.3$, $p<0.005$. These data indicate that the ethanol-dependent mice were tolerant to ethanol, and the tolerance (in total dips and total dip time) was still detectable on day 8 of withdrawal (Fig. 3B). In contrast, no cross-tolerance to CDP was detectable on either testing day in any of the three measures of head-dipping activity (Fig. 3A and B). Therefore, as revealed by data from the head-dipping test, chronic ethanol intake did not confer cross-tolerance to CDP.

DISCUSSION

Cross-tolerance among ethanol, other general depressants and opioids has been reviewed [14], but the relationship between ethanol tolerance and cross-tolerance to BZD is less well known. We have recently reported that ethanol-dependent mice are cross-dependent on CDP [2]. These data suggest that chronic ethanol treatment should confer cross-tolerance to CDP, be it in full or partial. Results presented in this study indicate the importance of using multiple behavioral tests to assess drug tolerance and cross-tolerance.

In the case of hypothermia, full cross-tolerance to CDP was observed in mice chronically treated with ethanol (Fig. 1). We tentatively concluded from results of the horizontal dowel test that full cross-tolerance to CDP was evident (Table 2). This was based on the comparisons between ethanol-dependent and control mice which showed similar percent increase in brain levels of ethanol (33%) and CDP (35%) in ethanol-dependent mice. Since CDP is a more potent drug than ethanol (differences in doses and brain drug levels), it is not surprising that the CDP fall-off time in the ethanol-dependent mice was much shorter than the ethanol fall-off time (Table 2). It should be noted that the ethanol fall-off time did not differ significantly from the CDP fall-off time in control mice. However, the conclusion that full cross-tolerance to CDP had developed may be equivocal because of the following: (1) The relationships between brain drug levels (ethanol or CDP) and fall-off times may not necessarily be linear, or they may not be identical when the two drugs are compared. (2) The rates of absorption and distribution of ethanol are not the same as those of CDP.

Only partial cross-tolerance to CDP was evident in the runway tests (Fig. 2), and virtually no cross-tolerance to CDP was detectable in the head-dipping test (Fig. 3); in the latter test, the only reliable measure of drug tolerance or cross-tolerance was the number of head-dips. Our results of the runway tests agree with those reported by Le *et al.* [15] who reported that prior treatment of ethanol conferred only partial cross-tolerance to CDP in rats. These investigators used a moving-belt and two-way shuttle-box avoidance tests to measure drug tolerance. Both tests required prior training of the rats. In contrast, none of the behavioral tests used in this study involved prior training of the mice. The horizontal dowel and runway test measured mainly a combination of the sedative and motor incoordinating effects of ethanol or CDP; it might also measure the effect of ethanol or CDP on exploration. The head-dipping test has the capability of measuring exploratory behavior independent of locomotor activity [9,17], although our apparatus did not have an independent measure of the latter. Therefore, lack of prior training in the behavioral tests was not a contributing factor to the non-equivalence between ethanol tolerance and CDP cross-tolerance.

Several factors may contribute to our observations of non-equivalence between ethanol tolerance and cross-tolerance to CDP. Kalant *et al.* [13] have stressed that tolerance does not necessarily develop at an equal rate to all

the actions of a given drug. Indeed, it has been shown that tolerance to BZD develops at very different rates for the various behavioral effects of BZD [8]. Likewise, Pohorecky *et al.* [20] have shown that tolerance to ethanol develops at different rates depending on the measures employed to evaluate it. Another important factor is that the rates of acquisition and dissipation of tolerance or cross-tolerance may not be the same for ethanol and CDP. Therefore, future investigations need to compare the rates of acquisition as well as dissipation of development of tolerance to ethanol, and cross-tolerance to CDP, and vice-versa. The neurochemical mechanisms underlying the developments of tolerance to ethanol and cross-tolerance to CDP have not been clearly delineated [8,24]. Therefore, it is premature to speculate on the neurochemical basis for the non-equivalence between ethanol tolerance and CDP cross-tolerance in ethanol-dependent mice.

The tolerance to ethanol and cross-tolerance to CDP determined in this investigation are primarily functional in nature. We have previously reported that chronic ethanol treatment using the same diet protocols did not induce metabolic tolerance to ethanol in the mice [6]. Although we have not investigated the effects of chronic ethanol treatment on the pharmacokinetics of CDP, the short time course of the behavioral tests (horizontal dowel test, 5 min; runway and head-dipping tests, 15–20 min; peak effect of ethanol or CDP hypothermia, 1/2 hr) render it unlikely that metabolic factors played an important role in the observed cross-tolerance to CDP.

In general, the test doses of ethanol and CDP used in this study yielded comparable pharmacological effects (as determined by the different behavioral tests) in control mice. There were two exceptions: (1) CDP-treated control mice had a longer latency for first run in the runway test on day 8 of withdrawal (Fig. 2B), compared to ethanol-treated control mice; (2) CDP-treated control mice had a longer latency for first head-dip in the head-dipping test on day 8 of withdrawal (Fig. 3B), compared to ethanol-treated or saline-treated control mice. We do not know the mechanisms for these differences. It should be noted, however, that there were no significant differences in these same parameters between CDP-treated control mice and CDP-treated ethanol-dependent mice.

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