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Dissociation of Tolerance and Physical Dependence After Ethanol/Chlordiazepoxide Intake[1]

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CHAN, A. W. K., D. L. SCHANLEY, F. W. LEONG AND D. CASBEER. *Dissociation of tolerance and physical dependence after ethanol/chlordiazepoxide intake.* PHARMAC. BIOCHEM. BEHAV. 17(6) 1239–1244, 1982.—The previously-observed attenuation of withdrawal reactions in mice (group B) fed an ethanol diet containing chlordiazepoxide (CDP) was not due to a difference in the rate of disappearance of blood ethanol levels during chronic diet treatment in group B compared to mice which received only the ethanol diet (group A). Injection of group A mice with CDP or N-demethyl CDP (10 mg/kg) at the time of diet withdrawal did not result in any significant attenuation of withdrawal scores. Injection of the lactam metabolite of CDP (LCDP; 10 mg/kg) resulted in significantly attenuated withdrawal scores at 4 and 6 hr only, but the pattern of withdrawal scores were different from that for group B mice. Moreover, blood LCDP level, in mice injected with LCDP, at 4 hr was at least five times higher than that attained in group B mice (from diet containing CDP). These results support our previous conclusion that the presence of major metabolites of CDP during withdrawal could only account for a minor contribution to the protective effect. Mice in A and B did not differ in the degree of functional tolerance which developed as a result of ethanol intake. Thus, there was an apparent dissociation between tolerance and physical dependence in the mice which had consumed the CDP/ethanol diet. The magnitude of decrease of GABA levels in the cerebellum and cerebral cortex at 4 hr after withdrawal also did not differ between the two groups, suggesting the reduction in GABA levels could not be correlated with the intensity of withdrawal signs.

Chlordiazepoxide Ethanol Withdrawal signs Tolerance GABA Physical dependence

WE have recently reported that C57BL/6J mice, which had been fed an ethanolic liquid diet containing chlordiazepoxide (CDP), showed less intense withdrawal reactions compared to mice which had been administered an ethanol diet alone [4]. Several factors may contribute to this phenomenon. These include: (1) the presence of CDP and/or its long-lasting metabolites may cause attenuation of withdrawal signs, since CDP and other benzodiazepines are used in the treatment of alcohol withdrawal [11,20]. (2) Chronic co-administration of CDP and ethanol may lead to an increase in the rate of metabolism of ethanol such that lower blood alcohol levels (compared to those in mice ingesting ethanol alone) were maintained. It has been demonstrated that the intensity of alcohol withdrawal reactions were directly correlated with blood alcohol levels during chronic ethanol intake [7]. (3) CDP might interfere with the mechanisms by which mice develop tolerance to and physical dependence on alcohol. (4) Chronic intake of CDP/ethanol might result in different neurochemical changes compared to those in mice which had received only ethanol. The neurochemical changes during withdrawal might be different in the two treatment groups, thereby leading to non-identical withdrawal manifestations. We have recently reported that the effects of an acute administration of CDP/ethanol on cerebellar c-GMP levels were significantly different from those resulting from the administration of ethanol alone [3].

The purpose of this study is to ascertain whether one or more of the above factors may be operative in causing the attenuation of alcohol withdrawal signs.

METHOD

Materials

Chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO). CDP was kindly provided by Hoffman-LaRoche, Inc. (Nutley, NJ). Chocolate-flavored Nutrament liquid diet was manufactured by Mead Johnson and distributed by the Drackett Products Co. (Cincinnati. OH).

Animals

Male C57BL/6J mice (8–9 weeks old) were purchased from the Jackson Laboratories, Bar Harbor, ME. They were

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housed singly in plastic cages in a controlled-environment room (21–22°) on a 12/12 hr light/dark cycle and received Teklad mouse diet (Teklad Mills, Winfield, IA) and tap water ad lib for 7 days before the beginning of an experiment.

Diet administration

Procedure for and duration of the administration of the ethanol diet with or without the supplementation of CDP was the same as those described previously [4]. The ethanol concentration in the alcohol diet was 3.5% for the first 6 days, and it was then increased by 1.5% every 3 days up to 8% [4]. In the earlier study, two different levels of CDP, namely, 3.2 or 6.4 mg/100 ml, were incorporated in the diet. However, results of withdrawal reactions were not significantly different in these two groups. Therefore in the present investigation, only the higher CDP level was used. A series of experiments involving similar schedules of diet administration were performed so that all necessary behavioral tests and chemical analyses (see below) could be accomplished. Basically each experiment involved four groups: Group A received the ethanol diet ad lib, the concentrations of ethanol being the same as previously described [4]; Group B received the ethanol diet containing CDP (6.4 mg/100 ml) ad lib; Group C was pair-fed (with A) an isocaloric diet with sucrose substituting for ethanol; Group D was pair fed (with B) the isocaloric diet containing CDP (6.4 mg/100 ml). Unless otherwise stated, mice were withdrawn from the ethanol diet at 8:30 a.m. on the 16th day of the start of administration.

Assessment of Tolerance to Ethanol

Two common measures were utilized, namely, ethanol sleep time and rectal temperature in response to a challenge dose of ethanol. (a) Ethanol sleep time: Mice in each group were injected with ethanol (4 g/kg, intraperitoneally, IP) at 3 hr after withdrawal of diet. This time was chosen because our previous work [4] indicated no detectable blood ethanol level in mice which had consumed the ethanol diet with or without CDP present. The challenge dose of ethanol effectively suppressed the withdrawal signs (e.g., convulsion on handling and body tremors) which would normally show up at 4 hr after diet withdrawal [4]. Goldstein [8] also reported that ethanol suppressed alcohol withdrawal reaction even when it was already underway. Procedures for the determination of sleep onset time and sleep time were the same as those described previously [19]. A blood sample was collected (see below) from each animal when it regained the righting reflex. (b) Rectal temperature: In a separate experiment, mice were injected with ethanol (3 g/kg, IP) at 3 hr after withdrawal of diet. Rectal temperature was measured [16] just before injection and at hourly intervals for 3 hr after injection. The same experiment was repeated one week after the first experiment, except that the dose of ethanol was increased to 3.5 g/kg. During that week, the mice received ordinary food pellets and water ad lib. This was to test whether tolerance to ethanol, developed as a result of ingestion of ethanol diet, had dissipated.

Measurement of Withdrawal Signs

As previously reported [4], two common measures were used, namely, rectal temperature [16] and withdrawal scores based on convulsions on handling [7]. These were determined in the absence of any challenge dose of ethanol. For withdrawal scores each mouse was assigned a score from 0 to 4 according to severity of convulsion elicited by handling; the same criteria as developed by Goldstein were followed [7].

Effects of CDP and Its Metabolites on Intensity of Withdrawal Signs

At the time of withdrawal of diet, mice which had consumed the ethanol diet only were injected (IP) with one of the following (N=8 to 11 for each treatment): CDP (10 mg/kg), N-demethyl-CDP (NDCDP, 10 mg/kg), the lactam derivative (demoxepam) of CDP (LCDP, 10 mg/kg) or saline. These doses were chosen such that the resultant blood levels of CDP and its metabolites would be comparable to or above those attained from the ingestion of ethanol diet containing CDP [4]. CDP and NDCDP were dissolved in saline containing 0.02N HCl, the latter was needed for solubilizing NDCDP, but LCDP was injected as a suspension in the above medium. The intensities of alcohol withdrawal signs were assessed at 2, 4, 6, 8, 11 and 13 hr after diet withdrawal.

Chemical Analyses

(a) Blood ethanol levels. In the experiment in which ethanol sleep time was determined, tail blood samples (10 μl) were collected when the mice regained the righting reflex. These were processed and analyzed for ethanol according to published procedure [13]. In another experiment, mice were injected with ethanol (3.5 g/kg, IP) three hours after withdrawal of diet and serial blood samples were taken at $^1/_2$, 1, 2, 3, 4, and 5 hr after injection. The rates of disappearance of blood ethanol level in different groups were computed from the linear regression analysis of the straight portion of the disappearance curve (at 2, 3, 4 and 5 hr).

(b) Blood LCDP levels. Blood samples were collected by heart puncture. LCDP was measured by a spectrofluorometric method of Koechlin and D'Arconte [12] as modified by Schwartz and Postma [18], with the exception that 300 μl of blood were extracted with 5 ml ether. Data relating to blood CDP and NDCDP levels after injection of the respective drugs are available from previous studies [2,9]; (see also Results section).

(c) γ-Aminobutyric acid (GABA) levels. Levels of this neurotransmitter were determined in three regions of the brain, namely, cerebellum, cerebral cortex and thalamus/hypothalamus. Mice were killed at $^1/_2$, 4 and 8 hr after diet withdrawal by immersion into liquid N_2 for two minutes. The preparation of brain extracts and the procedures for analysis of GABA were the same as those reported previously [1].

Statistical Analysis

Results were expressed as mean±S.E. Significance of the difference was analyzed by the Student's *t*-test or analysis of variance. The Mann-Whitney U test was utilized for ordinal measurements such as withdrawal scores.

RESULTS

Assessment of Ethanol Tolerance

Table 1 summarizes the responses to a challenge dose of ethanol (4 g/kg) by mice which had been exposed to the various diet treatments. Mice which had ingested the ethanol diet (Group A) showed a significantly prolonged sleep onset time and a significantly decreased sleep time compared to those which had been given the isocaloric sucrose diet

TABLE 1

EFFECT OF CHRONIC INTAKE OF ETHANOL DIET PLUS CHLORDIAZEPOXIDE (CDP) ON ETHANOL SLEEP TIME

Diet Treatment	Sleep Onset Time (min)	Sleep Time (min)	Blood Alcohol Level At Awakening (mg%)
Ethanol (A)	2.07 ± 0.07 ($p<0.001$ with C,D)	86.1 ± 7.5 ($p<0.05$ with B and C; $p<0.01$ with D)	285 ± 10 ($p<0.05$ with C and D)
Ethanol + CDP (B)	2.03 ± 0.15 ($p<0.005$ with C; $p<0.02$ with D)	66.5 ± 4.8 ($p<0.01$ with C; ($p<0.001$ with D)	280 ± 6 ($p<0.005$ with C; $p<0.02$ with D)
Isocaloric Sucrose (C)	1.34 ± 0.04	122.7 ± 12.9	245 ± 8
Isocaloric Sucrose + CDP (D)	1.51 ± 0.07	142.6 ± 17.1	251 ± 9

Mice were injected with ethanol (4 g/kg; IP) 3 hr after withdrawal of ethanol diet. N=8 to 10 in each treatment group.

(Group C) or the isocaloric diet containing CDP (Group D). Similar differences were obtained from mice which had consumed the ethanol diet containing CDP (Group B). The blood alcohol levels at awakening for Groups A and B were significantly higher than those for Groups C and D, indicating the development of tolerance in A and B.

The responses to the hypothermic effects of a single challenge dose of ethanol (3 g/kg) are depicted in Fig. 1a. Similar to the results of the sleep-time experiment, mice in Groups A and B, especially the latter, were less affected by ethanol than those in Groups C and D. The differences were more pronounced at one hour ($p<0.001$, compared to Groups C and D). Incorporation of CDP in the isocaloric sucrose diet did not render the mice in Group D to respond differently from those in Group C. When a higher challenge dose of ethanol (3.5 g/kg) was administered one week later, the maximal hypothermic effect was not achieved until about 2 hr (Fig. 1b). In this case, Groups A and B were significantly less affected than Groups C and D, especially at 2 and 3 hr ($p<0.001$, compared to Groups C and D), thus indicating that the formerly-acquired tolerance to ethanol had not dissipated during one week's abstinence from ethanol.

Alcohol Withdrawal Signs

In our previous investigation, we reported that at 2 1/2 hr after withdrawal, blood and brain samples from mice in Groups B and D showed no measurable level of CDP. There was also no detectable level of NDCDP in Group D; however, Group B showed a mean blood NDCDP concentration of 2.61±0.74 μg/ml (N=8) [4]. In this study, group B mice showed a mean blood LCDP level of about 0.15 μg/ml (N=4) at 4 hr after withdrawal; however, no detectable LCDP level (<0.1 μg/ml) was observed at 7 hr. The effects of injection of CDP or its metabolites on the severity of withdrawal signs in Group A mice (no CDP in ethanol diet) are shown in Fig. 2. Mice injected with CDP or NDCDP showed slightly lower withdrawal scores than those injected with saline, but the differences were not statistically significant. Mice injected with LCDP had significantly attenuated withdrawal scores

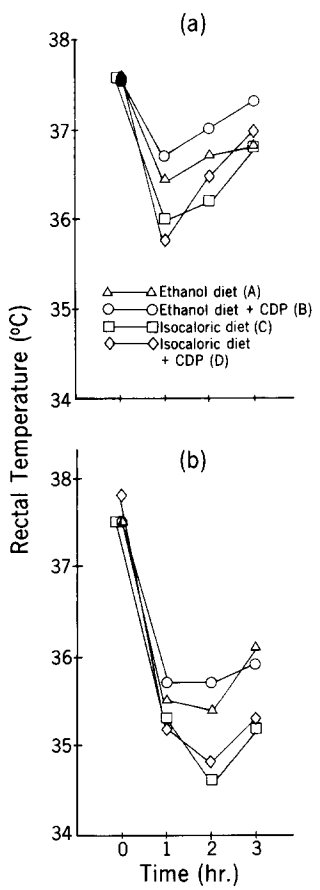

FIG. 1. Hypothermic responses to ethanol in mice after chronic intake of ethanol diet. Each point represents the mean value for 10 or 11 mice in each group. (a) Mice were injected with ethanol (3 g/kg) at 3 hr after withdrawal of diet. (b) Mice were fed ordinary food pellets and water ad lib for one week after diet withdrawal. They were then injected with ethanol (3.5 g/kg) on the following morning.

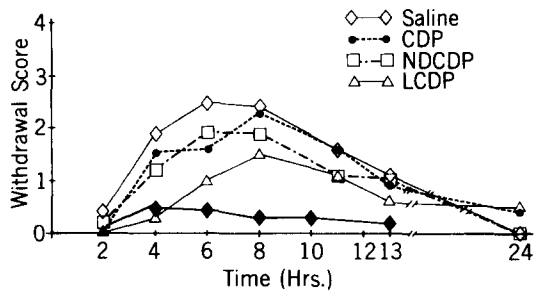

FIG. 2. Influence of CDP or its metabolites on the intensity of alcohol withdrawal scores. Mice were fed an ethanol diet for 15 days. At the time of withdrawal, mice were injected with one of the following: (1) Saline in 0.02 N HCl; (2) CDP (10 mg/kg) in 0.02 N HCl and saline; (3) NDCDP (10 mg/kg) in 0.02 N HCl and saline; (4) a suspension of LCDP (10 mg/kg) in 0.02 N HCl and saline. Each point represents the mean score for 7–11 mice in each group. ◆–◆ Withdrawal scores for mice which had consumed the ethanol/CDP diet and not injected with any drug at the time of withdrawal (data taken from [4]).

($p<0.05$) at 4 and 6 hr only, compared with mice injected with saline. We have previously shown that mice injected with CDP (10 mg/kg) did not have detectable blood level of CDP at 1 or 2 hr after injection, but blood NDCDP level exceeded 3 μg/ml for at least 4 hr [2,9]. Similarly, injection of NDCDP (10 mg/kg) resulted in blood NDCDP level being maintained between 2–3 μg/ml for at least 4 hr. Therefore, blood NDCDP levels resulted from injection of CDP or NDCDP were comparable to those in mice after chronic consumption of ethanol diet containing CDP (see above). In contrast, our present results indicate that Group A mice injected with LCDP (10 mg/kg) showed a mean blood LCDP level of 0.83±0.07 μg/ml (N=5) at 4 hr after injection. This was at least 5-fold higher than that determined in Group B mice (see above). Despite the higher blood LCDP levels in LCDP-injected mice, the withdrawal scores (except at 4 hr) were higher than those in Group B mice. These data suggest a minimal contribution from LCDP in attenuating the withdrawal scores.

TABLE 2

RATE OF DISAPPEARANCE OF BLOOD ALCOHOL LEVELS

Treatment Group	Disappearance Rate* (mg/ml/hr)
Ethanol Diet (A)	0.67 ± 0.04(N=10)
Ethanol Diet + CDP (B)	0.69 ± 0.06(N=11)
Isocaloric Control (C)	0.59 ± 0.05 (N= 8)
Isocaloric Control + CDP (D)	0.65 ± 0.07 (N= 7)

Mice were injected with ethanol (3.5 g/kg; IP) 3 hr after withdrawal of diet.

*From linear regression analysis of blood alcohol levels at 2, 3, 4 and 5 hr after injection.

Brain GABA Levels

Figure 3 summarizes data concerning brain GABA levels at three intervals after diet withdrawal. At 4 hr after withdrawal, mice in Groups A and B showed significantly decreased GABA levels in the cerebellum and cerebral cortex, compared to the corresponding values at 1/2 hr and to the values in Groups C and D at 4 hr (Fig. 3a and b). There was no significant difference in levels between Group A and Group B. Values in the thalamus-hypothalamus remained unchanged at 4 hr. GABA levels at 8 hr after withdrawal (Fig. 3c) returned to the same levels as those observed in 1/2 hr.

Ethanol Metabolism

Results depicted in Table 2 indicate that there was no significant differences in rates of disappearance of blood alcohol levels among the four treatment groups.

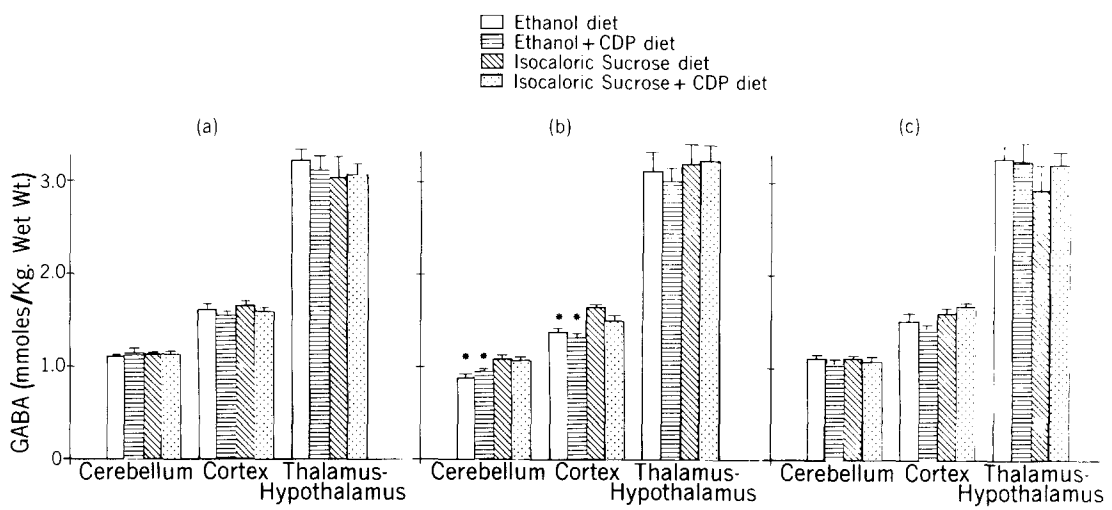

FIG. 3. Brain GABA levels during alcohol withdrawal. Mice were sacrificed at 1/2, 4 and 8 hr ((a), (b), (c), respectively) after diet withdrawal. Bars represent mean±S.E. and N=9 in each treatment group. *Significantly different from corresponding values at 1/2 hr and from values in the respective ($p<0.005$) control groups at 4 hr.

DISCUSSION

We have previously observed, at two time intervals after diet withdrawal, higher blood alcohol levels (BAL) in mice fed the ethanol diet alone (Group A) compared to those in mice fed the CDP/ethanol diet (Group B), although the difference was not statistically significant [4]. These data led us to speculate that there was an enhanced rate of ethanol metabolism in Group B mice. However, since we did not monitor the pattern of diet intake each day, we could not rule out the possibility that the feeding pattern might be different in the two groups, thereby leading to the apparent differences in BAL. At first glance the data in Table 1 for groups A and B seem to suggest that metabolic tolerance to ethanol developed in group B animals compared to group A, as reflected by the higher (though not significantly different) BAL of group A mice at a later awakening time. However, because of lack of data concerning peak ethanol levels achieved in individual mice and the variations inherent in sleep time determinations, the small difference (nonsignificant) in BAL at awakening between group A and group B cannot be used as reliable indicators concerning rates of disappearance of BAL. Therefore, a separate experiment was performed to test the rate of disappearance of BAL after a challenge dose of ethanol in the different treatment groups (A to D). Our results indicate no differences in the rates of disappearance of BAL in the four groups (Table 2). Therefore, the attenuation of alcohol withdrawal signs is not likely to be due to the maintenance of lower BAL (as a result of different rate of ethanol metabolism) in group B mice (compared to group A) during chronic ingestion of ethanol.

The injection of either CDP or NDCDP (10 mg/kg) in mice just before withdrawal of diet is shown to provide only a very minor protective effect on withdrawal scores (Fig. 2). This is in agreement with our previous conclusion that the presence of blood NDCDP during withdrawal could only account for a minor contribution to the protective effect [4]. In a preliminary report, Gessner and Hu [5] found that CDP was significantly more effective than saline in suppressing alcohol withdrawal reactions in mice. However, no detailed experimental data are available. Goldstein [8] reported that injection of CDP (20 mg/kg) five hours after alcohol withdrawal substantially reduced the severity of withdrawal reactions, in terms of peak height (maximum severity of the reaction), by 40% and area under curve, by 60%. The dose of CDP used was double that employed in the present study. Moreover, the time courses of withdrawal reactions were different between this study and Goldstein's investigation; the latter investigator used the inhalation method (plus injected pyrazole) to induce ethanol dependence in mice [8]. Therefore, a direct comparison of these results is not very meaningful. Our results indicate that injection of LCDP was more effective than CDP or NDCDP in lowering the withdrawal score, although the protective effect was only significant at 4 and 6 hr (Fig. 2). However, the mean blood LCDP level (0.83 μg/ml) at 4 hr after the injection of this metabolite were much higher than those (about 0.15 μg/ml or undetectable) resulted from the ingestion of the CDP/ethanol diet. These data do not support a significant contribution of LCDP in attenuating the withdrawal reactions in mice which had consumed the CDP/ethanol diet.

Results of this investigation clearly illustrate that functional tolerance to ethanol developed in both groups A and B mice (Table 1 and Fig. 1), and that the degree of tolerance was similar between these two groups. Therefore, CDP did not interfere with the development of ethanol tolerance. The sleep time data probably provide an underestimation of the degree of ethanol tolerance, because it has been reported [6] that the C57BL mice tended to become more sensitive to ethanol after repeated exposure to acute doses of the drug. However, the major difference in ethanol administration between the present study (liquid diet) and that (repeated injections) of Giknis *et al.* [6] might contribute to our observation of ethanol tolerance rather than increased sensitivity to alcohol. Our results are further supported by the other test of ethanol tolerance, namely, response to the hypothermic effects of ethanol. Since it is known that ethanol administration after withdrawal from alcohol effectively suppressed withdrawal signs [8], the hypothermic effect observed under this circumstance was due to the hypothermic effects of the injected ethanol, rather than that of withdrawal reaction. Had the withdrawal hypothermic effects been operative, group A mice would have shown a drop in rectal temperature to below 34° at 4 hr after diet withdrawal (equivalent to 1 hr after ethanol injection) [4]. However, the results shown on Fig. 1a do not suggest that this happened. If there was any confounding effect due to the withdrawal component, the degree of functional tolerance, as determined by the hypothermic effects of injected ethanol, would have been underestimated. The fact that we still observed the presence of functional tolerance in groups A and B one week after diet withdrawal (Fig. 1b) further supports the validity of the data shown in Fig. 1a. Thus, there seems to be an apparent partial dissociation between tolerance and physical dependence in the mice which had chronic intake of CDP and ethanol. A dissociation of tolerance and physical dependence has been reported by Ritzmann and Tabakoff [17], who found that C57BL mice pretreated with 6-hydroxydopamine before chronic ethanol treatment resulted in a block in development of tolerance, but the manifestation of physical dependence (e.g., withdrawal symptoms) were impeded. Our data suggest the opposite, namely, there was no block in tolerance development, but the manifestations of physical dependence were greatly attenuated.

Decreased brain levels of GABA have been observed in mice [14,15] and rats [21] in alcohol withdrawal. However, Hawley *et al.* [10] reported no change in GABA in the cerebrospinal fluid of patients undergoing alcohol withdrawal. Our results indicate that GABA levels in the cerebellum and cerebral cortex were significantly reduced at four hours after withdrawal in mice which had consumed the ethanol or CDP/ethanol diet (groups A and B; Fig. 3b), but there was no significant difference in the magnitude of decrease between the two groups. Thus the chronic intake of CDP together with ethanol did not affect this particular ethanol-induced neurochemical change during withdrawal. This suggests that the reduction in GABA levels could not be correlated with the intensity of withdrawal signs. However, the present data do not preclude the possibility that chronic intake of CDP/ethanol might produce different neurochemical effects (not determined in this study) compared to those caused by the intake of ethanol alone. Moreover, other neurochemical parameters during withdrawal might differ between groups A and B and these, coupled with the minor protective effect of NDCDP and LCDP, could account for the observed attenuation of withdrawal signs in group B.

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