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RELATIONSHIP OF BRAIN CYCLIC NUCLEOTIDE LEVELS AND THE INTERACTION OF ETHANOL WITH CHLORDIAZEPOXIDE*†

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Abstract—The effects of combined administration of ethanol (4 g/kg) and chlordiazepoxide (CDP, 12.5 mg/kg) on mouse brain c-AMP and c-GMP levels were investigated in order to test the hypothesis that the supra-additive effect of CDP on ethanol sleep time may be related to a supra-additive alteration in brain cyclic nucleotide levels induced by the combined drugs. Ethanol alone or CDP by itself did not cause any change in brain c-AMP levels, except for a transient decrease in the cerebral cortex and midbrain at 0.5 hr after ethanol injection, as well as a transient increase in the cerebellum at 0.5 hr after CDP injection. The combined drug treatment did not result in a supra-additive effect on c-AMP levels. On the other hand, c-GMP levels were depressed significantly for 4 hr after ethanol injection, especially in the cerebellum. The mice regained the righting reflex when the c-GMP levels were still about 30 per cent of control values. Ethanol and CDP together induced a supra-additive decrease of c-GMP concentrations in the cerebellum at 2 and 4 hr. This resulted in a lengthened period (about 2.5 hr) during which the cerebellar c-GMP levels were below 30 per cent of control values, and this interval coincided with the increase in sleep time, suggesting a possible relationship between these two factors. Injection of ethanol and *N*-demethyl-chlordiazepoxide (NDCDP) simultaneously (the latter being a metabolite of CDP) also elicited a more than additive depression of cerebellar c-GMP levels at 4 hr. These data suggest that NDCDP or its metabolite could be responsible for the supra-additive effect of CDP on the ethanol-induced decrease in cerebellar c-GMP levels.

Two of the benzodiazepines, diazepam and chlordiazepoxide, are among the most widely prescribed drugs in the United States. The consumption of both alcohol and a benzodiazepine is not uncommon. However, the biochemical and clinical consequences of their combined use have not been investigated thoroughly.

Several studies have shown that the combination of alcohol and benzodiazepines elicits additive or supra-additive effects [1–8]. We have recently reported [1, 2] that chlordiazepoxide (CDP) has a supra-additive effect on the duration of the ethanol-induced loss of righting reflex (sleep time) in mice. Such an effect was shown not to be due to alterations in the rate of ethanol metabolism in the animals treated with the combined drugs. However, it was found that brain CDP levels were higher in mice treated with ethanol and CDP than in those treated with CDP alone [1]. This is in line with the suggestion that the accumulation of the pharmacologically active desmethyl diazepam in the brains of ethanol-pretreated mice could explain the supra-additive effect of the ethanol–diazepam combination on motor coordination [6]. However, other central nervous system effects also must occur, since CDP, when injected alone (even at five times the highest dose used in the sleep-time study), did not cause the mice to lose their righting reflex.

The combined effects of ethanol and benzodiazepines on brain c-AMP and c-GMP levels have not been investigated. Several benzodiazepines are known to decrease cerebellar levels of c-GMP [9, 10] but not c-AMP [9]. Ethanol is also known to cause depression of cerebellar c-GMP in rats [11–13] and mice[14]. Its effect on mouse brain c-AMP seems to depend on the mouse strain and the brain region [14]. An acute dose of ethanol (3 g/kg) induced an increase in c-AMP level in the hypothalamus of BALB/cByJ (BALB) mice, but a small decrease in C57BL/6BY (BL/6) mice; a decrease of c-AMP level was observed in the cerebellum for both strains, but it was significantly more pronounced in the BL/6 mice than in the BALB mice [14].

This paper examines whether the time course of changes in brain cyclic nucleotide levels coincides with the duration of ethanol sleep time, and whether the supra-additive effect of CDP on ethanol sleep time may be related to a supra-additive alteration in brain cyclic nucleotide levels induced by the combined drugs. Another objective has been to compare

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the changes in brain cyclic nucleotide levels brought about by the combination of CDP–ethanol with those caused by the co-administration of ethanol and one of the metabolites of CDP, namely, *N*-demethylchlordiazepoxide (NDCDP), since it has been shown that the latter combination could account for the supra-additive effect of CDP/ethanol [2].

MATERIALS AND METHODS

Male C57BL/6J mice (9-weeks-old) were purchased from the Jackson Laboratories, Bar Harbor, ME. They were housed individually in plastic cages on a 12/12 hr light/dark cycle in a controlled environmental room (21–22°) and received Teklad mouse diet (Teklad Mills, Winfield, IA) and tap water *ad lib.* for 1 week before the start of the experiment.

CDP (the hydrochloride) and NDCDP were provided by Dr. W. E. Scott, Hoffmann–LaRoche, Nutley, NJ. In our initial investigation [1], CDP was injected 0.5 hr before ethanol injection, but we have since determined that the simultaneous injection of both drugs yields comparable sleep-time data [2]. Therefore, the more convenient method of simultaneous injection was chosen for the present investigation. In the first experiment, one group of mice was injected intraperitoneally (i.p.) with a solution of ethanol (20%, w/v; 4 g/kg) and CDP (62.5 mg/100 ml; dose 12.5 mg/kg) in saline. The dose of CDP was chosen because it was the highest dose we used in our sleep-time studies (other doses were 5, 7.5 and 10 mg/kg) [1]. It was rationalized that any alteration in brain cyclic nucleotide levels would be more clearly observed with the highest dose. Similarly, we used the same dose of ethanol as in our sleep-time experiments. Three other groups of mice were injected, respectively, with saline, saline/ethanol, and saline/CDP. Mice were killed at 0.5, 1, 2, 4 and 6 hr after injection. They were dropped into liquid N_2 and remained there for at least 2 min. Each frozen mouse was wrapped in aluminum foil and stored in a stoppered jar at −80° until used. Procedures of dissection of frozen brains have been described previously [15] and three regions were sampled, namely cerebellum, cerebral cortex and midbrain. The frozen tissue samples were homogenized at 4° in 1.0 ml of 6% trichloroacetic acid (TCA), and the extraction procedures for cyclic AMP and GMP were the same as those provided in the [^{125}I] RIA kits from the New England Nuclear Corp., Boston, MA. The two nucleotides were not separated by chromatography. The RIA kits were used for the radioimmunoassays of c-AMP and c-GMP, an acetylation procedure being incorporated for the analysis of the latter metabolite [16]. Pellets from the TCA extraction were used for protein determinations [17].

In a second experiment, one group of mice was injected with a freshly prepared solution of NDCDP (50 mg/100ml; dose 10 mg/kg) and ethanol (20%, w/v; dose 4 g/kg) in 0.02 N HCl. The use of dilute hydrochloric acid solution was necessitated by the solubility of NDCDP in this medium. The dose of NDCDP was chosen because our preliminary results indicated that blood levels of NDCDP resulting from this dose were very close to those resulting from injection of CDP. Collection, extraction and analysis of these samples were the same as those described previously [18]. Three other groups of mice were injected, respectively, with 0.02 N HCl, 0.02 N HCl/ethanol, and 0.02 N HCl/NDCDP. The mice were killed at 1, 2 and 4 hr after injection. The sampling of brain from these animals, the extraction of tissue samples, the analysis of c-GMP and c-AMP, and protein determination were performed as described above.

Procedures and criteria for the determination of ethanol sleep time were the same as those described previously [19].

Statistical evaluations of the data were performed by a computer program (BMDP2V) for analysis of variance.

RESULTS

Table 1 summarizes the ethanol sleep times that resulted from the injection of ethanol alone and in combination with CDP and NDCDP. It can be seen that an increase of about 165 min occurred in each of the cases where the combined drugs were administered.

Table 1. Ethanol sleep time after simultaneous injection of ethanol/CDP or ethanol/NDCDP*

Ethanol (g/kg)	CDP (mg/kg)	NDCDP (mg/kg)	Sleep time (min)
4			98.63 ± 5.41
4	12.5		263.60 ± 10.80†
4		10	265.10 ± 14.79†

* Values are means ± S.E.M.; N = 10 for each treatment.

† $P < 0.001$, compared to group injected with ethanol only.

With the exception of some transient changes (Table 2), there were no significant alterations in c-AMP levels in the cerebellum, cerebral cortex and midbrain at 1, 2, 4 and 6 hr after injections of CDP, ethanol, or CDP/ethanol. Table 2 summarizes the transient effects on c-AMP levels at 0.5 hr after injection. It can be seen that CDP caused a significant increase (18 per cent, $F = 18.14$, $P < 0.001$) of c-AMP in the cerebellum. On the other hand, ethanol significantly decreased c-AMP levels in the cerebral cortex (35 per cent, $F = 21.27$, $P < 0.001$) and midbrain (39 per cent, $F = 9.38$, $P < 0.005$). The combination of ethanol and CDP produced a significant interaction ($F = 9.10$, df 1,27, $P < 0.01$) only in the midbrain.

Figure 1 illustrates the effects of CDP, ethanol, or CDP/ethanol on brain c-GMP levels. No significant alteration occurred in the three brain regions at various times after CDP injections. On the other hand, ethanol consistently caused a significant depression of c-GMP levels, being more pronounced in the cerebellum than in the cerebral cortex or midbrain. The time course of the ethanol effect was also different in the cerebellum; at 0.5 hr after ethanol injection, the cerebellar c-GMP level was only about 20 per cent that of control and this gradually increased with time but was still significantly

Table 2. Regional brain cyclic AMP levels at 0.5 hr after combined ethanol and CDP administration*

Treatment	c-AMP levels (pmoles/mg protein)		
	Cerebellum	Cerebral cortex	Midbrain
Saline	8.33 ± 1.21	24.19 ± 1.16	19.58 ± 1.40
CDP	9.81 ± 0.73	21.63 ± 1.39	17.00 ± 1.14
	P < 0.001†	NS‡	NS
Ethanol	7.21 ± 0.58	15.55 ± 0.85	11.96 ± 0.71
	NS	P < 0.001†	P < 0.005†
CDP/ethanol	9.51 ± 0.80	17.03 ± 1.95	14.94 ± 1.53
	NS	P < 0.005†	P < 0.005† §

* Values are means ± S.E.M. (N = 8–10).
† Compared to saline-treated animals.
‡ NS, not significant.
§ Significant interaction effect (CDP × ethanol) from analysis of variance (P < 0.01).

depressed (about 60 per cent of control) at 6 hr. In contrast, the c-GMP levels in the cerebral cortex and midbrain were only moderately affected (about 70 per cent of control) at 0.5 hr after injection, and these levels decreased further at 1 hr to about 50 and 62 per cent of control for the cerebral cortex and midbrain respectively. Then a gradual recovery to control group values occurred between 2 and 6 hr; at the latter period, the c-GMP levels were not significantly different from control values. In the same two brain regions, the combined injection of CDP and ethanol resulted in a more pronounced (compared to ethanol injection) decrease in c-GMP levels at 2 and 4 hr. However, results of analysis of variance did not reveal a significant interaction effect. In the cerebellum, the decreases in c-GMP levels at 2 and 4 hr after CDP–ethanol injection were more than those induced by ethanol injection, and there was a significant interaction effect ($F = 4.76$, $df = 1,31$, $P < 0.04$ and $F = 5.58$, $df = 1,34$, $P < 0.02$ for 2 and 4 hr respectively). In other words, CDP/ethanol injection produced a more than additive effect in the depression of cerebellar c-GMP levels. Another analysis of variance (CDP × ethanol × time) also yielded a highly significant interaction in the cerebellum ($F = 4.38$, $df = 4,145$, $P < 0.002$), but not in the cerebral cortex or midbrain.

The contribution of one of the metabolites of CDP, namely NDCDP, in inducing some of the

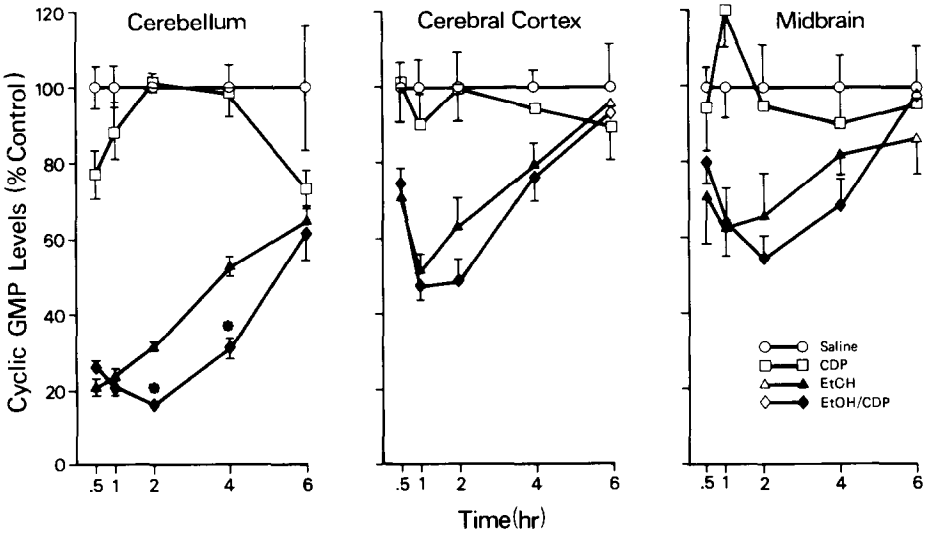

Fig. 1. Effects of combined ethanol and chlordiazepoxide (CDP) on regional brain cyclic GMP levels. Mean values for each time period from saline-treated animals (controls) are set at 100 per cent, and those from other treatments are expressed as percentages of the respective controls. Each point is the mean value (± S.E.M.) from eight to ten animals for each time period. Closed symbols indicate significant differences (P < 0.05) from saline-treated animals, and an asterisk (*) next to one of these symbols denotes a significant interaction effect (CDP × ethanol) from analysis of variance. Representative values for saline-treated mice at 0.5 hr are 6.72 ± 0.35, 1.74 ± 0.11 and 1.22 ± 0.07 pmoles/mg protein for the cerebellum, cerebral cortex and midbrain respectively.

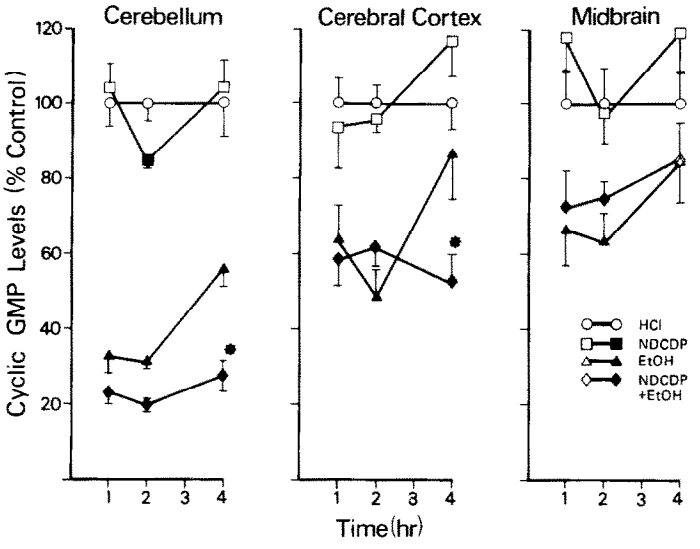

Fig. 2. Effects of combined ethanol and *N*-demethyl-chlordiazepoxide (NDCDP) on regional brain cyclic GMP levels. Each point, expressed as a percentage of the respective control value (level from HCl-injected mice set at 100 per cent), is the mean value (± S.E.M.) from eight to ten animals for each time period. Closed symbols indicate significant differences ($P < 0.05$) from saline-treated animals, and an asterisk (*) next to one of these symbols denotes a significant interaction effect (CDP × ethanol) from analysis of variance. Representative values for mice injected with 0.02 N HCl at 1 hr are 3.38 ± 0.22, 1.66 ± 0.11 and 1.23 ± 0.10 pmoles/mg protein for the cerebellum, cerebral cortex and midbrain respectively.

neurochemical changes mentioned above was investigated by injecting mice with ethanol–NDCDP. The blood levels of NDCDP resulting from such an injection were comparable to those attained after CDP/ethanol injection, e.g. at 2 and 3 hr after injection, NDCDP levels (μg/ml) were 3.05 ± 0.48 and 2.62 ± 0.14 (N = 8), respectively, for NDCDP/ethanol, and 2.29 ± 0.14 and 3.18 ± 0.23 (*N* = 8) for CDP/ethanol injection. When compared with the vehicle of injection (0.02 N HCl), NDCDP, ethanol, or ethanol/NDCDP did not significantly alter c-AMP levels in the three main brain regions. The c-AMP levels in the three brain regions of mice injected with 0.02 N HCl were comparable to those obtained from saline injection (Table 2). Cyclic GMP levels were also not affected by NDCDP injection, except in the cerebellum at 2 hr only (Fig. 2). The effects of ethanol injection on c-GMP levels in this experiment were almost identical to those observed in the previous experiment (Fig. 1), indicating reproducibility of the effects as well as chemical measurements. Cerebellar c-GMP levels were 20–30 per cent of controls between 1 and 4 hr after ethanol/NDCDP injection (Fig. 2). Like the combination of ethanol and CDP, NDCDP/ethanol produced a more pronounced effect on c-GMP levels in the cerebellum than in the cerebral cortex and midbrain. The depression of c-GMP levels in the cerebellum by the combination of NDCDP and ethanol was more than that induced by ethanol alone. Significant interaction effects were observed at 4 hr in the cerebellum ($F = 5.93$, $df = 1,28$, $P < 0.02$) and cerebral cortex ($F = 5.67$, $df = 1,25$, $P < 0.03$) (Fig. 2). Another analysis of variance (ethanol × NDCDP × time) also revealed a significant interaction in the same two brain regions (cerebellum, $F = 3.69$, $df = 2,84$, $P < 0.03$; cortex, $F = 4.54$, $df = 2,79$, $P < 0.02$). There was no significant difference in the magnitude of decreases in cerebellar c-GMP by ethanol/CDP or ethanol/NDCDP injections, although the trend was for a lesser effect in the midbrain by ethanol/NDCDP.

DISCUSSION

There seems to be a general agreement among investigators that ethanol decreases cerebellar c-GMP levels. Our results offer another confirmation and further indicate that other parts of the brain are similarly affected, albeit to a lesser extent. However, conflicting reports have appeared concerning the effects of ethanol on brain c-AMP levels in rats [20–22] and mice [23, 24]. Our results indicate that ethanol alone or CDP by itself did not cause major changes in brain c-AMP levels, except for a transient decrease in the cerebral cortex and midbrain 0.5 hr after ethanol injection, as well as a transient increase in the cerebellum 0.5 hr after CDP injection. The combined drug treatment did not result in a supra-additive effect on c-AMP levels. In fact, a less than additive effect was observed at 0.5 hr only in the midbrain. Our data are in agreement with those reported by Church and Feller [14] who found decreases of c-AMP levels in the cerebellum and hypothalamus 30 min after an acute dose of ethanol in C57BL/6BY mice (compared to the C57BL/6J mice used in this investigation). These authors also indicated that there was a strain difference in the brain cyclic AMP response to ethanol [14]. It has been reported that Swiss albino mice also show a

decrease in cerebellar c-AMP levels after being exposed to alcohol vapor for 7 days [23]. However, increases of cerebellar and subcortical c-AMP levels following acute doses of ethanol in C57BL/Ka mice have been reported [24].

Our results on ethanol sleep time show that mice injected with 4 g/kg ethanol usually lost the righting reflex for about 1.5 hr (Table 1). Since the ethanol-induced reduction in c-GMP levels lasted more than 4 hr in the different brain regions (Fig. 1), it can be inferred that the decrease in c-GMP levels was probably more related to the general intoxicating effect of ethanol, rather than specifically correlated with the sleep time. Mice injected with ethanol alone regained the righting reflex while the cerebellar c-GMP levels were still depressed (about 30 per cent of controls). At that time, the blood ethanol levels were still quite high (usually around 250–400 mg/100 ml) [19]; therefore, the lower c-GMP levels reflected the persisting CNS effects of ethanol. The mean sleep time for mice injected with ethanol/CDP was about 4.3 hr, and it can be seen from Fig. 1 that mice treated this way also regained the righting reflex when the cerebellar c-GMP levels were about 30 per cent of controls. The levels of c-GMP at awakening were, therefore, alike for mice injected with ethanol or ethanol/CDP, although the time course was different for these two groups. The same phenomenon also occurred in the midbrain, although the c-GMP concentration at awakening for either treatment was about 65 per cent of controls (Fig. 1). However, in the cerebral cortex, the c-GMP level at awakening for ethanol-treated mice was lower than that for mice injected with ethanol/CDP. Thus, the cerebellum was more affected by ethanol, as well as by ethanol-CDP, than the cerebral cortex and midbrain. We have observed a more than additive effect of CDP/ethanol on c-GMP levels at 2 and 4 hr only in the cerebellum (Fig. 1). The increased duration (about 2.5 hr) of c-GMP levels being depressed below 30 per cent of control values coincided with the increase in ethanol sleep time, suggesting a possible relationship between these two factors. It is speculated that the two drugs interact near membrane receptors which, in turn, leads to alterations in cyclic nucleotide levels. Further investigations are needed to delineate the mechanisms of this interaction.

We have demonstrated that when mice were injected with both *N*-demethyl-chloridazepoxide (NDCDP) and ethanol, the former being a metabolite of CDP, the increase in ethanol sleep time was similar to that induced by CDP/ethanol injection (Table 1). The plasma levels of NDCDP in the mice injected with NDCDP/ethanol were comparable to those that resulted from injection of CDP/ethanol (unpublished results). These data implicate a role of NDCDP or its metabolites in the apparent supra-additive effect of CDP on ethanol sleep time. Results presented in this paper indicate that NDCDP or its metabolites may also be responsible for the supra-additive effect of cerebellar c-GMP levels induced by CDP/ethanol injection (Figs. 1 and 2). It has been reported that in mice the antipentylenetetrazol activity appeared to parallel the brain levels of NDCDP rather than those of CDP or its lactam metabolite (LCDP) [25]. In the monkey, the taming effects appear to parallel the concentration of the parent compound in the brain rather than those of the metabolites [26]. Our results do not preclude the possibility that LCDP, a metabolite of NDCDP, may contribute to the supra-additive effects (from CDP/ethanol injection) on sleep time and cerebellar c-GMP levels. Preliminary results from this laboratory indeed suggest that the combination of LCDP and ethanol also produces a supra-additive increase on ethanol sleep time. The extent to which this metabolite is involved in the combined actions of CDP and ethanol awaits further investigation.

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