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Ethanol Stimulation After Chronic Exposure in C57 Mice

LAWRENCE D. MIDDAUGH, JEAN P. FAVARA AND WILLIAM O. BOGGAN

Medical University of South Carolina, Department of Psychiatry & Behavioral Sciences
Room 803 Research Building, 171 Ashley Avenue, Charleston, SC 29425-0742

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MIDDAUGH, L. D., J. P. FAVARA AND W. O. BOGGAN. *Ethanol stimulation after chronic exposure in C57 mice.* PHARMACOL BIOCHEM BEHAV **34**(2) 331–335, 1989.—The effects of chronic exposure to ethanol via an ethanol containing diet on locomotor activity and on the response to depressive and stimulatory doses of ethanol were examined in C57BL/6cr (C57) mice. Mice maintained for approximately 3 weeks on a liquid diet in which 25% of the calories derived from ethanol had blood levels ranging from undetectable to 60 mg% when sampled at 2200 hr. They were less active than nutritionally equivalent controls, were less depressed by a high dose of ethanol (3 g/kg), and were more stimulated by a low dose of ethanol (2 g/kg). The results establish that chronic EtOH exposure via liquid diets attenuate its depressive effect and exacerbate its stimulatory effect on locomotor activity of C57 mice as previously shown for other strains or for rats. The increased activity elevation in chronically exposed mice may reflect an unmasking of excitation upon developing tolerance to EtOH depression.

C57 Mice Locomotor activity Ethanol Tolerance Ethanol stimulation

NUMEROUS studies indicate that ethanol has a dose-related biphasic action of stimulation and depression [see (14) for review]. Whether these opposing effects are due to quantitative differences in ethanol effects on the same mechanism or to its effect on separate mechanisms is unclear. However, the changes which occur in the depressive and the stimulatory effects of ethanol during chronic exposure appear to be quite different. Mice become tolerant to the depressive, but not to the excitatory effects of ethanol (4, 10, 11, 17). In addition, some studies indicate that as mice become tolerant to ethanol depression, its excitatory action is unmasked (4, 10, 11). In these studies, ethanol was given chronically for long periods of time in the drinking water or via once or twice daily injections. One report, however, indicated that tolerance to ethanol hypnosis produced by an alcohol-containing liquid diet did not unmask the drug's excitatory effect in three mouse strains (17). The C57BL/6 (C57) mice used in this study were not stimulated by the low dose of ethanol, whether they were naive or tolerant to the hypnotic effects of the drug. The lack of ethanol stimulation in naive C57 mice confirmed several other reports for this strain (2, 7, 9, 15). The lack of EtOH stimulation in mice tolerant to its hypnotic effects observed in the study was interpreted by the authors (17) as evidence against the hypothesis that increased sensitivity to ethanol depression which in turn masks its activating effect accounts for the absence of stimulation frequently noted for this strain. This interpretation, however, is compromised by several methodological characteristics of the study which reduce the probability that ethanol-induced stimulation could be detected in tolerant mice. First, the activity monitors used in the study do not clearly differentiate between locomotor and other types of motor behavior (e.g., rearing). Since a drug-induced increase in locomotion could be counter-balanced by a reduction in rearing, the activity monitor used might be less sensitive to the stimulatory effects of ethanol. Second, hypnosis rather than reduced locomotion was the measure of ethanol depression; and it is possible that tolerance to the hypnotic effect of ethanol is mediated by a different mechanism than tolerance to its locomotor depression effect. Third, tolerance to alcohol was established by feeding an ethanol-containing liquid diet, hence, was not associated with the additional cues associated with daily or twice-daily injections. Finally, the ethanol dose, although maximally stimulatory to DBA and BALB mice, may not be the most appropriate dose for detecting changes in the stimulatory effects of the drug after chronic exposure.

Although C57 mice are not readily stimulated by ethanol, our laboratory (12,13) and others (4,6) have established conditions under which the drug stimulates this strain. Since previous work has established the conditions favorable for observing ethanol stimulation in C57 mice, we decided to reassess whether mice with chronic exposure to ethanol via the dietary method would show evidence of tolerance to the depressive effects and an increased stimulatory effect of ethanol. A smaller second experiment was conducted to determine blood levels of ethanol produced by the dosing procedure used in the first experiment and to determine if the mice maintained on the diet showed any evidence of withdrawal between the time of removing the diet and behavioral testing.

METHOD

Experiment 1

Female C57BL/6cr mice (Wilmington, MA) 60 days old at the beginning of the experiment were used. The mice were maintained on a light:dark cycle of 0700:1900 in a colony room

contiguous with the behavioral laboratory. They were housed individually and fed vitamin- and mineral-fortified Sustacal (Mead-Johnson, Evansville, IN) liquid diets. One group (E), 42 mice, was given a diet containing sufficient ethanol to account for 25% of the total calories ingested (4.4% v/v 95% EtOH). A second group (S), 42 mice, was given a diet which was nutritionally equivalent to the ethanol diet, but 25% of the calories derived from sucrose. The sucrose-enhanced diet was given in amounts equivalent to laboratory norms for mice ingesting the ethanol diet (i.e., 17 ml). These diets provided the only source of liquid and nutrition for 21 days. Diets were provided fresh daily in 40-ml graduated conical centrifuge tubes which could be read to the nearest 0.5 ml. The liquid diets were replaced with standard lab chow (Wayne Rodent Blox) and water for 24 hr prior to testing. The amounts of diet consumed were recorded for approximately half of the animals in each group to insure that consumption for these mice conformed to laboratory norms.

All testing was completed between 1300 and 1500 hr (22–24 hr after removing the diet). Mice were tested individually in one of three activity runways enclosed in light- and sound-controlled, ventilated chambers. The runways were constructed of clear Plexiglas and were oval shaped with inside and outside wall dimensions of 19×33 and 31×45 cm, respectively. The runway floor was 6 cm wide and the walls were 18 cm high. Photocells and opposing infrared light sources were located at four equidistant points around the runway at a height of 1.25 cm above the floor. Interruption of the light source was detected, amplified and recorded to define one unit of activity. Electronic programming was such that an adjacent photocell needed to be activated before reactivating any given photocell. Thus, a unit of activity was equivalent to locomotion of at least one quarter the distance around the runway, 32 cm.

On the day of testing, animals from the two diet conditions were brought to the laboratory, weighed, and injected IP (0.02 ml/g body weight) with either vehicle (distilled water) or ethanol (2.0 g or 3.0 g/kg). The animals were then immediately placed individually into one of the three activity monitoring apparatuses and activity was recorded in a dark environment for 15 min. Activity counts were cumulated and printed out at 5-min intervals. The individual treatment conditions were distributed equally across the three runways. The runways were cleaned of fecal material and urine, wiped with disinfectant, and dried after each test to eliminate olfactory cues from previous tests.

The experimental design for the experiment was a 2 (Diet Condition) × 3 (Acute EtOH Dose) × 3 (Test Time) factorial with repeated measures on the Time factor. Five of the 6 groups created by this design contained 14 mice each. Two of the mice maintained on the ethanol diet and tested with the 3 g/kg dose were eliminated from the analysis; one because it did not drink the diet, and the other because of equipment malfunction.

Experiment 2

Female mice of the same age and strain described above were used. Six mice were maintained for 25 days on the ethanol diet and 8 were pair-fed the sucrose diet as described above. Beginning from a reference point of 0800 hr on the 25th day of diet exposure, we determined the amounts of diet consumed and ethanol in blood samples taken from the orbital sinus (75 μl into heparinized capillary tubes) at 2200 and again at 0800 hr the following day. Previous reports (1) and unpublished data from our laboratory indicated that EtOH levels peak near 2200 hr for C57 mice maintained on EtOH diets. Aliquots (50 μl) of the blood samples were transferred to 15 ml tubes containing 0.5 ml of 1.2 N perchloric acid, capped with a rubber stopper, and refrigerated until assay the following day. The amounts of ethanol in the samples were determined by incubating the tubes for 20 min in a shaking water bath at 37°C and then injecting 1 cc of the head space into the injection port of a Gas Chromatograph (Shimadzu Model GC4B) containing a Chromosorb 101 column and a flame ionization detector. Temperatures for the injection port, column, and detector were 280, 125, and 120°C respectively. Amounts of EtOH in the samples were established by comparing peak heights with those from standards with known amounts of EtOH.

Evidence for withdrawal was assessed by determining body weight, rectal temperature, and the presence of seizure activity when the diet was removed (i.e., 0800 hr after 25 days on the diet) and at 3, 6, 9, and 24 hr after removal. Seizure activity was assessed by observing the animal for the presence of clonic or tonic seizures when lifted from the cage by the tail and rotated 180 degrees as described (8). Temperatures were assessed using a digital thermometer (Bailey Instruments, Inc., Model TH-5) with a probe inserted to 2.5 cm. Temperature was recorded after a stable reading for 15 sec.

RESULTS

Experiment 1

Amounts of diet ingested (ml) averaged over the last three days for the two groups (mean ± SE) were 16.9 ± 0.4 for the S-group and 16.2 ± 0.3 for the E-group. This slightly reduced consumption (4.1%) for the E-group was not supported statistically, $t(49) = 1.456$, $p = 0.148$. Body weight at the time of testing was 3.7% lower for mice maintained on the ethanol than on the control diet [mean ± SE: S-group, 21.7 ± 0.2; E-group, 20.9 ± 0.2; $t(80) = 2.438$, $p = 0.02$].

Data from the activity tests are summarized in Table 1 and were initially analyzed with the analysis of variance (ANOVA) appropriate to the design. The results of this ANOVA indicated that activity level depended on the complex interaction of Diet Condition, EtOH Dose, and Time after injection, $F(4,152) = 4.766$, $p<0.001$. Of particular interest was that maintenance on the ethanol diet influenced the acute effect of the ethanol injections [Diet × Dose Interaction: $F(2,76) = 4.213$, $p = 0.018$]. Inspection of the scores for the two vehicle groups in the table indicates that chronic exposure to ethanol itself lowered locomotor activity. These data were analyzed with a separate ANOVA (Diet × Time) which indicated that the distribution of activity across time was different for the two groups, $F(2,52) = 9.432$, $p<0.001$. Analysis of the simple main effects indicated significant reductions in activity for the E-group compared to the S-group during the first two 5-min intervals of testing.

Because the vehicle controls differed in locomotor activity, the influence of chronic ethanol exposure on the acute effects of the two ethanol doses was assessed on data normalized to the vehicle control group for each diet condition. The normalized data expressed as percent increases or decreases from vehicle controls at each time period are shown for both ethanol doses in Fig. 1. An ANOVA on these data indicated that the effects of the different doses depended on the prior diet condition, $F(1,50) = 5.607$, $p = 0.02$. The 2 g/kg dose of EtOH produced greater increases in activity of animals maintained on the ethanol diets compared to sucrose controls at all time periods, $F(1,52) = 13.549$, $p<0.001$. The effect of the 3 g/kg dose of ethanol depended on an interaction of time and prior diet condition, $F(2,48) = 5.297$, $p = 0.008$. Mice maintained on the chronic ethanol diet were less depressed by this dose than were sucrose controls during the early part of the testing period.

Experiment 2

Mice ingested similar amounts of the sucrose (16 ml) and the

TABLE 1

EFFECTS OF CHRONIC AND/OR ACUTE ETHANOL ON LOCOMOTOR ACTIVITY (MEAN ± SE) OF FEMALE C57BL/6cr MICE*

	Chronic Ethanol Dosing					
	Sucrose Diet			Ethanol Diet		
Postinjection Interval (min)	Acute Ethanol Dosing (g/kg) 0	2	3	0	2	3
5	82 ± 10	100 ± 11	40 ± 6	56 ± 5	123 ± 3	56 ± 6
10	59 ± 8	70 ± 9	32 ± 5	42 ± 3	97 ± 10	28 ± 6
15	39 ± 6	51 ± 7	27 ± 5	38 ± 3	64 ± 9	25 ± 5

*Activity during a 15-min test depended on a complex interaction of chronic ethanol condition, acute ethanol condition and time, $F(4,152) = 4.766$, $p<0.001$. Compared to sucrose controls, mice maintained on an ethanol diet (4.4% v/v) for 21 days were less active after vehicle injection, more stimulated by the low EtOH dose, and less depressed during the first time period after the high EtOH dose. See text for statistical analyses.

ethanol (15 ml) diets over the 24-hr period during the last day of the diet. The amount of ethanol in blood of individual mice ranged from undetectable to 60.2 mg% (mean = 33.1 mg%) at 2200 hr and was not detectable in any of the animals at 0800. None of the mice showed evidence of seizure activity at any of the times tested. The temperature and body weights of mice at intervals after removing the diets are shown in Table 2. Body weight for both groups at time 0 in Table 2 was the same as measurements taken at 2200 hr the night before. As noted in the table, mice maintained on the E diet were initially heavier than controls but they lost weight after being switched to lab chow and water and were not different from controls by 9 hr after terminating the diet. This difference in body weight change across time for the two groups is supported by a significant interaction of Diet Condition × Time [$F(4,48) = 13.308$, $p<0.001$, and subsequent analysis of the simple main effects, $p<0.05$]. Body weight for the E-group declined systematically over the 24 hr after removing the diet with statistically significant reductions at each time point ($p<0.01$, Duncan's Test). Although temperature appears to be elevated for mice withdrawn from ethanol on the morning of diet removal and 24 hr later, these differences were not statistically supported.

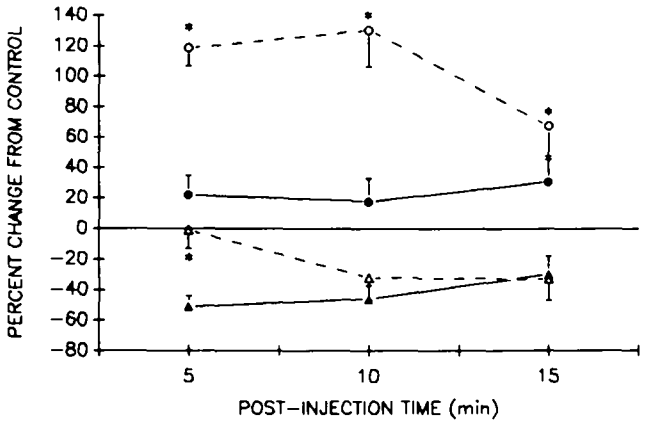

FIG. 1. Ethanol-induced increases or decreases (mean ± SE) in locomotor activity compared to vehicle controls for mice injected with 2 (circles) or 3 g/kg (triangles) doses of ethanol. Mice were maintained for 21 days on a diet containing ethanol (4.4% v/v, open symbols) or equivalent amounts of an isocaloric sucrose diet (filled symbols). Asterisks indicate statistically significant differences between the two diet groups.

DISCUSSION

Compared to nutritionally equivalent sucrose controls, C57 mice tested 24 hr after discontinuing an ethanol-containing diet had reduced locomotor activity, an attenuated reduction in activity produced by a high ethanol dose (3 g/kg), and greater stimulation to a low ethanol dose (2 g/kg).

The reduced locomotor activity for mice maintained on the chronic ethanol diet and injected with vehicle prior to testing is consistent with a previous report (17) in which the dietary ethanol exposure was at a higher concentration (7% vs. 4.4% v/v), but for a shorter duration (7 vs. 20 days). This activity reduction in C57 mice chronically exposed to EtOH appears to be robust in that it occurred whether the measure was restricted to locomotion as in our study or included other forms of motor activity as in the previous study. The EtOH levels in blood obtained with the dosing procedure used in our study (<70 mg% for any animal) were much lower than the 100–300 mg% reported for the dosing procedure used in the previous study. The reduced activity observed in these studies was not due to residual EtOH since both tests were given 24 hr after removal of the diets and blood ethanol could not be detected on the morning of diet removal in our study and by 6 hr after removal for the earlier study.

Since the animals in both experiments showed evidence of tolerance and were tested 24 hr after discontinuing the diets, the

TABLE 2

BODY WEIGHT AND TEMPERATURE DURING A 24-HR PERIOD AFTER DISCONTINUING LIQUID DIETS CONTAINING SUCROSE (S) OR ETHANOL (E)

Time After Diet Removal (hr)	Body Weight (g)*		Temperature (°C)	
	S	E	S	E
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
0	22.8 ± 0.4	24.4 ± 0.6†	37.5 ± 0.2	38.1 ± 0.1
3	22.5 ± 0.5	23.9 ± 0.6‡	37.7 ± 0.1	37.7 ± 0.1
6	22.4 ± 0.4	23.6 ± 0.6‡	37.7 ± 0.1	37.8 ± 0.1
9	22.3 ± 0.4	23.3 ± 0.5	37.2 ± 0.1	37.6 ± 0.2
24	22.3 ± 0.4	22.9 ± 0.5	37.8 ± 0.2	38.2 ± 0.1

*ANOVA summary: [Diet × Time: $F(4,48) = 13.308$, $p<0.001$].
†E > S, $p<0.05$, Duncan's test.
‡E > S, $p<0.10$, Duncan's test.

contribution of alcohol withdrawal to these results must be considered. Although withdrawal from EtOH is commonly associated with increased activity or reactivity rather than the activity reductions noted in the two studies, hypoactivity during EtOH withdrawal has sometimes been reported (5). Evidence of withdrawal (i.e., spontaneous seizures or hypothermia) has been reported for C57 mice upon terminating the 7% EtOH dosing procedure described above (16). These signs, however, were maximal at 6 to 8 hr after discontinuing the diet and had dissipated by 24 hr. Mice maintained on the EtOH diet in our study showed no evidence of spontaneous seizure activity or hypothermia at any time over the 24-hr period examined. The possibility of subtle withdrawal effects remains, however, because of the 6% weight loss for ethanol exposed mice during this 24-hr period.

The activity reduction produced by the high EtOH dose was not as extensive in chronic ethanol-exposed mice (20%) as for sucrose controls (45%). This difference was greatest during the first period of testing when activity of drug-injected mice was reduced by 51% for sucrose controls, but was similar to vehicle controls for mice chronically exposed to EtOH. The ultimate effect of a sufficiently high dose of EtOH on motor activity is a product of its biphasic excitatory and depressive action with excitation being most apparent during early periods after injection (12). Chronic ethanol-exposed mice in the present study, thus, show some tolerance to the depressive effects of EtOH as reflected in a delayed onset of the depressive phase of its biphasic action. If one assumes that hypnosis is an extreme form of EtOH depression, the present result is consistent with the previous report that C57 mice maintained on the ethanol diet develop tolerance to its hypnotic (depressive) effects.

Although the results of the present study were consistent with the only other study on the stimulatory and depressive effects of EtOH after maintenance on an EtOH diet (17), the present study differs from the previous report in that chronically exposed mice injected with the low EtOH dose had elevated locomotor activity. Methodological differences between the two studies most likely account for the different results. The dose of ethanol used in the previous study (1.35 mg/kg) had no influence on motor activity in either control or chronically exposed mice. This dose, although maximally stimulatory for the BALB and DBA strains, was probably not optimal for C57 mice. Ethanol stimulation in C57 mice is not a robust phenomenon and reliably occurs only under restricted conditions (3, 4, 12, 13). Doses of 1.0 g (12) and 1.33 g/kg (4) reportedly stimulate C57 mice only under special conditions of light and when assessed at critical postinjection times. The EtOH doses for the present study were chosen on the basis of previous work indicating that they clearly depressed (3 g/kg) or minimally stimulated (2 g/kg) activity of C57 mice (12) under the testing conditions used in the present experiment. The failure of the 2 g/kg ethanol dose to elevate locomotion of sucrose control mice in the present study is consistent with previously reported results for C57 mice maintained on lab chow (12). In the earlier study, the 2.0 g/kg dose elevated activity when the animals were tested in the light, but not in the dark as in the present study. It is possible that our use of this borderline stimulatory dose allowed us to detect increased ethanol stimulation in animals maintained on the ethanol diet in the present study, whereas it was not in the previous report (17).

The increased stimulatory effect of EtOH in mice chronically exposed to the drug via the diet confirms previous reports in which chronic exposure was by repeated daily injections (10) or ethanol in drinking water (11) of Swiss Webster mice, and by once or twice daily injections of ethanol into C57 and DBA mice (4). Thus, chronic EtOH exposure, whether by repeated injections or via the dietary method, exacerbates its stimulatory effects in C57 mice as noted for other mouse strains. The increased stimulatory effect of EtOH in chronically exposed mice could be the consequence of hyper-reactivity to the ethanol injections because of subtle withdrawal effects or perhaps due to an unmasking of the EtOH excitation as the animals become tolerant to its depressive effects as suggested in earlier studies (4, 10, 11). Although the mechanism is unclear, the present study clearly establishes that chronic exposure to EtOH via the dietary method exacerbates its stimulatory effects in C57 mice as previously noted for other mouse strains and for rats.

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