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Exacerbation of Ethanol Withdrawal Seizures in Mice With a History of Multiple Withdrawal Experience

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BECKER, H. C., J. L. DIAZ–GRANADOS AND R. L. HALE. *Exacerbation of ethanol withdrawal seizures in mice with a history of multiple withdrawal experience.* PHARMACOL BIOCHEM BEHAV **57**(1/2) 179–183, 1997.—Repeated ethanol withdrawal experience has been shown to result in an exacerbation of future withdrawal episodes. This sensitization of the withdrawal response has been hypothesized to represent a "kindling" phenomenon. We previously demonstrated that mice exposed to ethanol vapor for a total of 48 h exhibited more severe withdrawal seizures if the exposure was divided into three 16 h intoxication/8 h abstinence cycles than if the 48 h of exposure occurred in a single bout. The present study was designed to further characterize this model of ethanol withdrawal "kindling" and determine whether such a "kindled" response may be evident when withdrawal testing is conducted after an additional bout of intoxication that is the same for all groups. Adult C3H mice were chronically exposed to ethanol vapor in inhalation chambers for 40 h prior to withdrawal testing. Prior to this 40 h intoxication period, one group (Multiple Withdrawal; MW) received three cycles of 16 h ethanol vapor separated by 8 h abstinence; a second group (Single Withdrawal; SW) did not receive any ethanol exposure prior to the 40 h test cycle; a third group (Continuous Exposure; CE) received the same total ethanol exposure as the MW group (48 hr), but without interruption; and a control group (C) did not receive any ethanol treatment throughout the experiment. Blood ethanol levels following the 40 h bout of ethanol intoxication were 100–140 mg/dl for all ethanol-exposed groups. The severity of handling-induced convulsions during withdrawal was significantly greater in the MW group compared to CE and SW groups. These results suggest that differences in the severity of ethanol withdrawal seizures due to differences in prior withdrawal experience can be demonstrated even when later ethanol exposure patterns are equated. As such, the results provide further support for the "kindling" hypothesis of ethanol withdrawal. © 1997 Elsevier Science Inc.

Ethanol Withdrawal Kindling Seizures Mice

IT IS NOT uncommon for alcoholics to experience several ethanol withdrawal syndromes which result from periods of abstinence during the course of their chronic abusive drinking (12). A number of clinical and experimental studies have indicated that a history of multiple ethanol withdrawal experiences may increase the severity of future withdrawal episodes. Ballenger and Post (1) hypothesized that this progressive intensification of the withdrawal syndrome following repeated episodes of ethanol intoxication and withdrawal may represent the manifestations of a "kindling" mechanism (similar to that described for electrical stimulation of discrete brain areas (10)). That is, it was postulated that each episode of CNS hyperexcitability that normally accompanies ethanol withdrawal may serve as a stimulus supportive of a "kindling" process. This "kindling" or sensitization process then, may underlie the commonly observed progression of withdrawal symptoms, from mild responses characteristic of initial withdrawal episodes (irritability, tremors) to more severe symptoms associated with subsequent withdrawal syndromes such as seizures and delirium tremens (1).

A growing body of clinical and experimental findings has provided support for the "kindling" hypothesis of ethanol withdrawal. For example, clinical studies have found that patients with histories of previous detoxifications were more

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likely to experience a seizure or other withdrawal-related complications during an index detoxification than patients without such histories (5,6,14). Similarly, animal studies have demonstrated an increase in neural hyperexcitability and severity of ethanol withdrawal symptoms following repeated withdrawal experience (7,13,15,16,17,18). This work has further substantiated clinical studies supportive of the "kindling" hypothesis of ethanol withdrawal.

We have established an animal model of ethanol dependence that is sensitive to the effects of prior withdrawal experience (2,4). In this model, mice are repeatedly intoxicated and withdrawn from chronic ethanol exposure delivered by the inhalation route. Following a 16 h bout of ethanol intoxication, withdrawal seizures were significantly more severe in animals that had prior intoxication/withdrawal experience than animals that were withdrawn for the first time. Thus, a single bout of ethanol intoxication (16 h) that yielded an initially mild withdrawal response was found to result in a more severe withdrawal reaction (convulsions) when such experience was repeated. Moreover, this exacerbated withdrawal response was observed even when the total amount of ethanol exposure was equated across groups (4). That is, mice exposed to ethanol vapor a total of 48 h exhibited more severe withdrawal seizures if exposure was divided into three 16 h intoxication/8 h abstinence cycles than when the 48 h of exposure occurred in a single continuous (uninterrupted) bout. Further, a positive relationship was demonstrated between the severity of withdrawal seizures and the number of previously experienced withdrawal episodes (2). Importantly, blood ethanol levels were similar for all ethanol-exposed groups just prior to withdrawal assessment (2,4).

The purpose of the present study was to further characterize this model by examining whether an exacerbated ("kindled") withdrawal response may be still evident when withdrawal testing is conducted after an additional bout of intoxication that is similar for all ethanol-exposed groups. That is, in previous work, whereas continuously-exposed and multiple withdrawal groups were tested for withdrawal seizure severity following the same total amount of ethanol exposure, the former group was tested following 48 h of exposure while the latter group was tested after a (final) 16 h bout of intoxication. Thus, the present study was designed to evaluate whether the potentiated withdrawal response may be observed even when testing is conducted after an additional bout of intoxication that is identical for all groups (excluding non-ethanol exposed controls). In this way, differences in the severity of withdrawal seizures among the various groups may be attributed to differences in previous withdrawal histories.

METHODS

Subjects

Adult male C3H/He mice (80 to 100 days of age) purchased from Charles River Laboratories (Portage, MI) were used as subjects. The animals were housed three to four per cage in an AAALAC-accredited facility under a 12 h light/dark cycle (lights on at 0600), with lab chow food and water continuously available.

Study Design and Experimental Procedure

Mice were randomly assigned to one of four groups (N = 15–19/group), as described in Fig. 1. One group of mice (multiple withdrawal; MW) received three cycles of 16 h continuous ethanol vapor exposure in an inhalation chamber separated by 8 h periods of abstinence. Following the third intoxication/withdrawal cycle, the mice were exposed to an additional 40 h bout of intoxication and then tested for their withdrawal response. A second group (single withdrawal; SW) was treated identically as the MW group. However, prior to the 40 h bout of ethanol exposure, SW mice were placed in a control chamber (in the absence of ethanol) during the three 16 h periods when MW mice were exposed to ethanol vapor. The third group (continuous exposure; CE) received the same total amount of ethanol exposure as the MW group, but prior to the final 40 h bout of intoxication, 48 h (16h × 3) of ethanol vapor treatment was administered to these mice in a continuous rather than intermittent fashion. The fourth group of mice (C) served as controls and were maintained in the control chamber throughout the experiment. Withdrawal testing was conducted for all mice at the same time. Further, the MW, SW, and CE groups were all tested following an identical 40 h bout of ethanol exposure. However, the groups differed in their prior experience with ethanol (the MW group received three prior episodes of withdrawal, the SW group had no prior experience with ethanol, and the CE group received the same amount of ethanol exposure as the MW group, but were tested after a single withdrawal experience that separated the 48 h and 40 h bouts of intoxication).

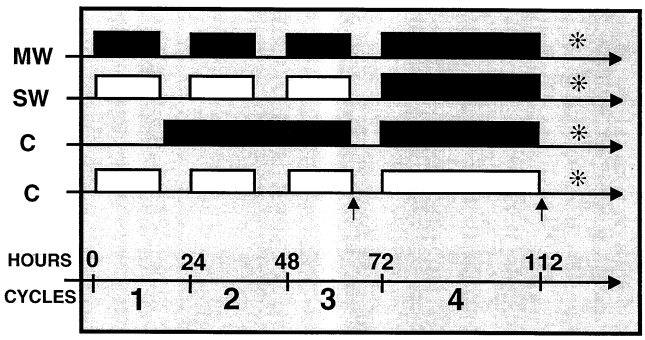

FIG. 1. Schematic diagram of study design. Solid bars represent time spent in the ethanol inhalation chamber while open bars represent time spent in the control (air) chamber. Arrows indicate when blood samples were taken from all animals and the asterisk indicates when withdrawal testing (HIC) was conducted.

Chronic Ethanol Administration

Ethanol was chronically administered by the inhalation route, as previously described (4). Briefly, mice were placed in inhalation chambers (60 × 36 × 60 cm) modified after Goldstein (11); the housing conditions in these chambers were identical to that in the colony room. Ethanol (95%) was volatilized and delivered to one of the chambers at a rate of 140–180 μl/min by a peristaltic pump (Harvard Apparatus). This, in combination with air being delivered to the chambers at a rate of 10 l/min, maintained the ethanol concentration in the chamber in the range of 10–13 mg/l air (mean ± SEM: 11.01 ± 0.14 mg/l).

At the beginning of each dependence cycle (1700 h), intoxication was initiated by administration of ethanol (1.6 g/kg; 8% w/v) and blood ethanol concentration (BEC) was stabilized by injection of the alcohol dehydrogenase inhibitor pyrazole (1 mmol/kg). Both drugs were injected IP in a volume of

0.02 ml/g body weight. Mice being placed in the control (air) chamber were given an initial loading dose of saline rather than ethanol. These mice also received an injection of pyrazole. The CE group received an injection of ethanol upon initial entry into the ethanol chamber and pyrazole injections at the same time as the other groups. During the final 40 h ethanol intoxication bout, all animals were briefly removed from the chambers and given an injection of pyrazole. In this way, all mice received the same number of pyrazole injections prior to withdrawal testing. Withdrawal testing was conducted 24 h after pyrazole administration and at the same time for all groups (see Fig. 1).

As indicated in Fig. 1, immediately after removing the mice from the chambers at a time corresponding to the third withdrawal cycle for the MW group (0900 h), blood samples were collected from all mice (including controls) for subsequent blood ethanol analysis. Blood samples were collected again upon final removal of the mice from the chambers. Following this second blood collection, all mice were individually housed and coded (tail marked with ink) for dependence testing.

Withdrawal Seizure Assessment

Withdrawal severity was assessed by scoring handling-induced convulsions (HIC). The HIC scoring scale, as depicted in Table 1, was modified after Crabbe and Kosobud (8) and Goldstein (11). The HIC measure has proven to be a useful and reliable index of CNS hyperexcitability associated with ethanol withdrawal, and in particular, multiple ethanol withdrawal experience (2,4). During the final withdrawal phase, all mice were scored for HIC hourly for the first 10 h and then at 24 h post-withdrawal. Withdrawal testing was conducted by a single experimenter that was blind to the animals experimental history. Data are presented as hourly HIC scores and area under the 24 h withdrawal curve.

Ethanol Samples and Measurement

Chamber ethanol concentration was monitored twice daily (at 0900 and 1700 h). Air samples from the inhalation chambers (3 ml) were collected with a 5000 μl Hamilton gastight syringe through a port in the chamber wall. The air samples were then transferred to Venoject tubes for later analysis. Blood samples (10 μl) were collected from the retro-orbital sinus with heparinized capillary tubes at times indicated in Fig. 1. The samples were diluted 50:1 with 3.4% perchloric acid (v/v), vortexed, and then centrifuged at 12,000 × g. The supernatant was analyzed using a spectrophotometric enzymatic assay previously described (4). Blood ethanol concentrations were expressed as mg/dl blood and chamber ethanol concentrations were expressed as mg/l air.

TABLE 1

HANDLING-INDUCED CONVULSION (HIC) RATING SCALE

Score	Description of Behavior
0	No activity on tail lift, or after gentle 360° spin
1	No activity on tail lift, but facial grimace after 360° spin
1.5	Facial grimace on tail lift
2	Tonic convulsion after 360° spin
3	Tonic/clonic convulsion after 360° spin
4	Tonic convulsion on tail lift
5	Tonic/clonic convulsion on tail lift, onset delayed by 1 to 2 s
6	Severe tonic/clonic convulsion on tail lift, no delay in onset
7	Severe tonic/clonic convulsion prior to tail lift

TABLE 2

BLOOD ETHANOL CONCENTRATIONS AT WITHDRAWAL (mg/dl)*

Group	*N*	Prior to 40 h Test Cycle	Following 40 h Test Cycle
MW	18	160.06 ± 7.5	112.17 ± 8.54†
SW	19	6.11 ± 1.45	133.68 ± 3.81
CE	16	161.00 ± 3.90	135.38 ± 2.70
C	15	9.00 ± 1.20	5.80 ± 0.53

*Values represent mean ± SEM. †Significantly different from SW and CE groups ($p < 0.01$).

Data Analysis

Blood ethanol levels and withdrawal HIC data were analyzed by analysis of variance (ANOVA), with post-hoc comparisons (Fisher's Protected Least Significant Difference Test) conducted when appropriate.

RESULTS

BECs prior to and following the final 40 h bout of ethanol exposure for each of the treatment groups are presented in Table 2. As can be seen, BEC did not significantly differ between MW and CE groups prior to the final 40 h exposure period (SW and C groups did not receive ethanol treatment up to this point). At the time of final withdrawal (following the 40 h intoxication bout), BECs were significantly lower for

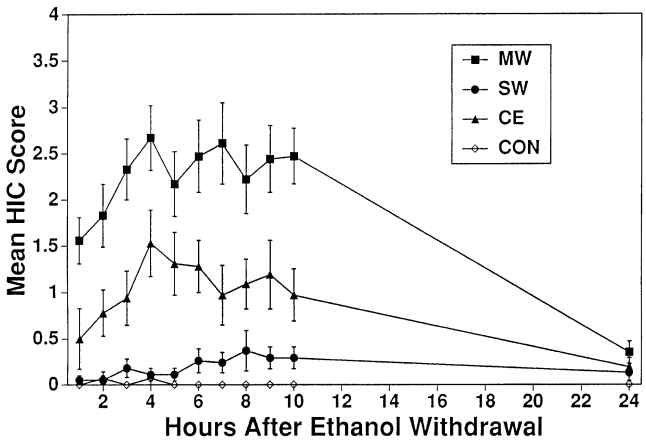

FIG. 2. Mean ± SEM. HIC scores for each withdrawal treatment group as a function of time following final withdrawal.

the MW group in comparison to SW and CE groups [$F(2, 50) = 5.11$, $p < 0.01$].

The progressive development of HIC during the withdrawal testing period for each of the treatment groups is illustrated in Fig. 2. As can be seen, the severity of withdrawal HIC was greatest for the MW group, intermediate for the CE group, and minimal for the SW group. The incidence of spontaneous (non-ethanol related) convulsions was negligible (C group). This is supported by ANOVA which revealed a significant main effect of Treatment [$F(3, 640) = 520.66$, $p < 0.001$]. Further, repeated measures ANOVA revealed a significant Treatment × Time interaction [$F(30, 640) = 55.66$, $p < 0.001$], which indicated that the group differences were evident at most time points until the withdrawal response subsided at 24 h post-withdrawal, at which time there were no group differences.

As a measure of the severity of the overall withdrawal response, the area under the 24 h HIC curve was calculated for each subject and presented in Fig. 3. ANOVA revealed a significant effect of treatment condition [$F(3, 64) = 36.33$, $p < 0.001$]. Subsequent analysis indicated that all ethanol-exposed groups differed significantly from the C group. Moreover, as depicted in the figure, the MW group evidenced the most severe withdrawal response followed by the CE and SW groups, with each group significantly different from the others ($p < 0.05$).

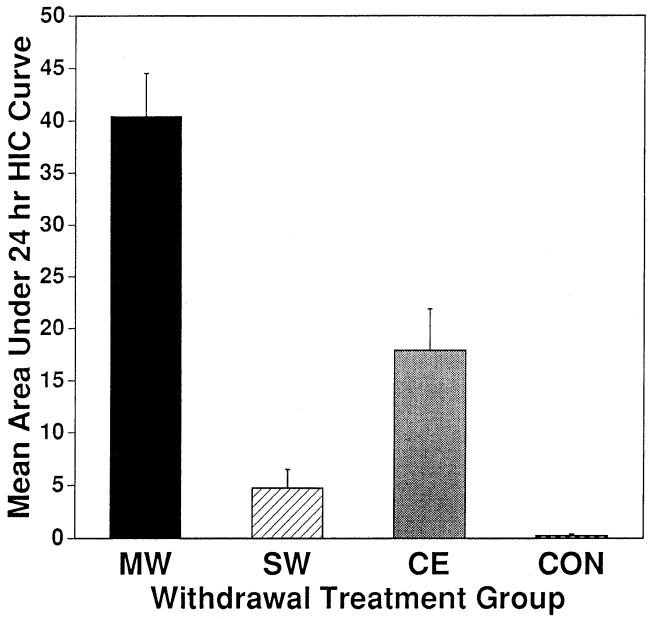

FIG. 3. Mean ± SEM area under the 24 h ethanol withdrawal HIC curve for each treatment condition.

DISCUSSION

Results from this study demonstrate that multiple ethanol withdrawal experience increases the severity of withdrawal seizures during a subsequent withdrawal episode. Further, differences in the severity of withdrawal seizures due to differences in prior withdrawal experience were demonstrated even when later ethanol exposure was equated prior to withdrawal assessment (MW, SW, and CE groups were tested following an identical 40 h bout of ethanol intoxication). Moreover, even when total amount of ethanol exposure was equated prior to the 40 h bout, the intensity of withdrawal seizures was more severe in animals that were administered chronic ethanol in a discontinuous pattern (with intervening periods of abstinence) in comparison to those that were exposed to ethanol in a continuous (uninterrupted) fashion (compare MW and CE groups in Figs. 2 and 3). Of course, a true continuously exposed group (88 h ethanol exposure) was not included in the study design. Thus, differences between MW, CE, and SW groups during the final test withdrawal reflect comparisons between animals having had previous experience with 3, 1, and 0 withdrawal cycles, respectively. Nevertheless, these results indicate that not only total amount of ethanol exposure (dose and duration), but a history of withdrawal experience, may influence and contribute to the intensity of a later withdrawal response. As such, these results support our previous findings in which a similar mouse model was employed (2,4), as well as lend further support to the "kindling" hypothesis of alcohol withdrawal.

Importantly, differences in the severity of withdrawal seizures among groups with different prior withdrawal experience can not be attributed to differences in the level of intoxication immediately preceding withdrawal assessment. In fact, withdrawal seizures were more intense in the MW group than the SW and CE groups, despite the fact that the MW group evidenced significantly lower BEC at the time of withdrawal (Table 2). In addition, previous work has shown that the exacerbation of withdrawal seizures in mice with multiple withdrawal experience is not related to differences in the rate of ethanol elimination upon final withdrawal (2). Thus, it would appear that the potentiated withdrawal response is not related to pharmacokinetic factors but rather, an alteration in neural excitation.

The relatively mild withdrawal response exhibited by the SW group in the present study was somewhat surprising given that the animals were tested following a 40 h bout of exposure. In our previous work, a group of mice tested after continuous exposure to 48 h of ethanol vapor exhibited a greater withdrawal (HIC) response (3). The difference in withdrawal response between these groups may be related to the 8 h difference in duration of exposure, or the fact that in the previous study, significantly higher BEC were achieved in comparison to that obtained in the present study. It is likely that both factors are involved in this difference in intensity of the withdrawal response (ethanol dose and duration of exposure).

Although the mechanism(s) underlying this withdrawal sensitization phenomenon are yet to be fully elucidated, a number of studies provide support for the notion that repeated ethanol withdrawal experience results in a progressive intensification in CNS hyperexcitability. For example, electroencephalographic studies have demonstrated more intense EEG changes associated with a second withdrawal episode in comparison to that following the first withdrawal experience (17,18). In addition, our laboratory (9) and others (13) have shown that animals with multiple withdrawal experience exhibit greater sensitivity to (pro)convulsant drugs, as well as facilitated chemical (3) and electrical kindling (16). Clearly, additional studies are needed to identify neural changes related to the potentiated withdrawal seizure response.

In summary, results from this study demonstrate that repeated ethanol withdrawal experience results in an exacerbation of subsequent withdrawal seizures. Further, this potentiated withdrawal response was evident even when total amount of ethanol exposure as well as the final bout of intoxication just prior to withdrawal assessment was equated among groups.

Although the mechanisms underlying the phenomenon are unknown, these results suggest that history of prior detoxifications should be considered an important determinant in predicting the severity of future withdrawal episodes, as well as an important factor in developing strategies for treatment and long-term management of chronic alcohol abusers.

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