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Behavioral and Physiological Measures for Studying Ethanol Dependence in Mice[1]

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HUTCHINS, J. B., D. L. ALLEN, L. S. COLE-HARDING AND J. R. WILSON. *Behavioral and physiological measures for studying ethanol dependence in mice.* PHARMAC. BIOCHEM. BEHAV. **15**(1) 55–59, 1981.—In the initial experiment, 20 male and 20 female heterogeneous stock (HS) mice were divided randomly into experimental and control groups. Ethanol was administered in liquid diet form to the experimental group in increasing dosages for a 9-day period followed by behavioral and physiological tests to determine differences between groups and genders 6 to 7 hr post-withdrawal. Analysis of data showed significant differences between the ethanol-treated and control groups for at least six of the measures. In addition, significant gender differences and significant interactions between gender and treatment group were found for four of the measures; in all of these cases, females appeared to exhibit a less severe withdrawal syndrome than males. In a second experiment, no gender difference in rate of ethanol disappearance from the blood could be found in 36 HS mice tested at 1 to 3 hr post-withdrawal.

Alcohol Ethanol Ethanol dependence Ethanol disappearance rate Gender differences Mice Withdrawal syndrome

RESEARCH into the basis of human alcoholism has recently focused on genetic explanations for differences in human susceptibility to alcohol addiction. Among the most useful of the animal models for studying these genetic differences are those which utilize withdrawal measures in rodents. Several quantitative methods for measuring the severity of alcohol dependence in rodents, especially mice, have been developed. The use of these measures hinges on the assumption that the severity of withdrawal symptoms is directly related to the degree of dependence; this assumption has been borne out consistently, not only in rodents (e.g., [10]) but in humans as well (e.g., [18]). These studies have shown that longer periods of intoxication result in more severe withdrawal symptoms. Seizure scores [7, 9, 10, 11, 12] and rectal body temperatures [6,29] are among the most reliable and well-documented measures of alcohol dependence, and they have been used rather extensively in rodent studies. However, all but one of these experiments have used only one gender; the exception is a selection study. This design eliminates any possibility of finding significant gender differences which may also be a factor in human alcoholism.

Studies by McClearn and Rodgers, beginning some 20 years ago, have indicated a genetic basis for alcohol preference in mice [21, 22, 30]. Later studies, notably those of Goldstein [11], used seizure scores to show that some differences in alcohol dependence between mice are attributable to genetic factors. Seizures are among the most obvious and consistent signs of alcohol withdrawal in both humans and rodents [7, 10, 11, 12, 18, 25]; loss of hypothalamic regulation of body temperature is also frequently observed in the withdrawal syndrome. At low room temperature (4°C), mice show marked hypothermia after ethanol withdrawal, while hyperthermia is observed at 34°C [29].

Although they have not been widely reported in the literature, it is our belief that activity measures can also be utilized to quantify the extent of ethanol dependence in mice. Marked hypoactivity has been observed in male rats 5 to 9 hr post-withdrawal [17,26], while one other study has reported little effect on activity in male rats tested 14 to 15 hr post-withdrawal [32].

Effects of gender on ethanol dependence have not been fully investigated, although studies of the effects of gender on rates of ethanol disappearance from the blood in C57BL mice [3] and demonstrations of ethanol effects on the hypothalamic-pituitary-gonadal axis in male rats [2] suggest possible areas of further study.

There are similarities between symptoms of ethanol withdrawal in humans (described in [18]) and the ethanol withdrawal signs in mice described below and in other reports [7, 10, 11, 12]. In addition, genetic factors may be involved in human alcoholism [1, 15, 16].

Thus, it was our intention in initiating this study to de-

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velop a consistent, reliable set of measures for determining degree of ethanol dependence in mice following chronic administration and to examine gender differences, if any, in these measures and in rate of ethanol disappearance from the blood.

METHOD

Experiment 1

Twenty male and 20 female HS mice [23] maintained in the Institute for Behavioral Genetics specific-pathogen-free laboratory were assigned randomly to either an ethanol-diet or a control-diet group. The subjects were housed individually in metal cages with wood shavings as bedding. Tap water was provided ad lib; liquid diet was available through inverted 25-ml graduated cylinders with glass sipper tubes extending into the cage. Feeding began at 0700 or 0830 hr for each group of subjects; the first day on the liquid diet was designated Day 1 of the treatment period. The mice were fed Lieber-DiCarli liquid diet (Iso-Cal, Bio-Serv, Inc. #711-PRA for the ethanol-treated group, and #711-PRC for controls). Ethanol-treated animals received increasing concentrations of alcohol as follows: On Days 1 and 2, 95% ethanol was added to the ethanol-type diet to produce a concentration of 10% ethanol-derived calories (e.d.c.); on Days 3 and 4, the concentration was 20% e.d.c.; and on Days 5 through 9, 35% e.d.c. Such a progressive schedule allowed the mice to become dependent upon ethanol.

Experimental and control subjects were matched by sex and by weight (as closely as possible) prior to the experiment and were pair-fed to assure equal consumption of liquid diet (and parallel weight loss) in control and ethanol-treated groups. After 9 days on the respective diets, each ethanol-group mouse received an IP injection of 12.5% ethanol in saline solution according to the following formula: (3.4 ml − amount of 35% e.d.c. diet consumed during the 6 hr prior) × (0.0089) × (body weight in grams) = ml 12.5% ethanol solution injected. This formula was devised to raise blood ethanol levels to approximately 300 mg% at the time of withdrawal for ethanol-group mice. Control-group mice received a sham injection.

No correction was made for spillage of the liquid diet. A "dummy" set up to test for leakage showed that average leakage was approximately 3.1 ml/day. A large number of sipper tubes were available, and those showing obvious leakage were removed immediately. The tubes were used randomly for all mice, and it is assumed that errors due to leakage are distributed randomly across conditions.

In order to minimize experimenter bias, subjects were tested without *a priori* knowledge of their group status. The mice were 83 to 85 days old at the time of testing. The animals were tested in groups of 10 beginning at 1300 or 1430 hr on Day 10 of the study. Tests are described below in the order that they were performed.

Seizure scores. The scale used for seizure scores is a modification of the 5-point scale described by Goldstein [10, 11, 12]. A score of 3 indicated spontaneous seizures (in the absence of external stimuli, other than usual laboratory sounds and dim lighting). A score of 2 indicated a seizure elicited by lifting the mouse by the tail. A score of 1 was recorded if seizures occurred after a gentle 360° turn while the mouse was being lifted by the tail. A score of 0 indicated that no seizures occurred using these procedures.

Hole-in-wall. This apparatus was originally described in connection with earlier open-field activity and emotionality studies [4]. It is a Plexiglas box, 31.8 × 31.0 × 15.2 cm high, which contains an X-shaped barrier with a hole, 4.2 cm in diameter, in the center of each of the four walls, 1.0 cm from the floor. Two opposing compartments are lighted through translucent Plexiglas floors; the other two compartments are kept dark. The subject was placed in a lighted compartment, confined in a transparent cylinder (9.5 cm diameter × 12.9 cm high) for 90 sec to adjust to the new surroundings, and then released for a 3-min test. Scores were recorded for number of crossings (forepaws touching the floor of an adjacent compartment), vicarious trial entries (inserting the head into an adjacent compartment through the hole without entering), rearing (lifting the forepaws off the floor), time of latency to first crossing, total time spent in dark compartments, seizures and their severity, urination before and during testing, and defecation before and during testing.

Body temperature. Rectal temperatures were taken using a DigiTec Model 4810 electronic thermometer with a flexible lubricated probe inserted to a depth of 2.2 cm at a room temperature of 25 ± 0.5°C. Rather than holding the animals while their temperatures were taken (which might have affected their body temperature), each mouse was restrained in a clear Plexiglas cylinder (4.3 cm in diameter) with a slot that extended along the length of the tube and across one end. The experimenter gently lifted the mouse's tail through the slot, positioned the animal at the end of the tube, and touched only the tail while the temperature was being taken.

Vertical screen. This apparatus consists of a 1.22 m × 2.44 m piece of hardware cloth (3.2 mm mesh) mounted vertically on a wooden frame. The screen is divided into 5.1 cm squares, and a foam-rubber "crash pad" is placed underneath the screen in case the mouse falls. At the start of the test, the mouse was placed in the middle of the screen and allowed to roam freely for 3 min. During this time, the number of times the mouse moved from one square to another, number of approaches to each side, time spent at top, number of jumps off the screen, number of fecal boli, and presence or absence of urination were recorded.

Scores for each of the 21 measures on the four tests were compared using a two-way analysis of variance of the effects of treatment and gender.

Experiment 2

Thirty-six HS mice (18 males, 18 females) were tested for the rate of disappearance of ethanol from the blood. Samples were taken at 1 and 3 hr post-withdrawal for 19 of the subjects (group 1); the remaining 17 subjects (group 2) were tested at 1, 2 and 3 hr after withdrawal. Feeding and injection procedures were exactly as described above.

Blood samples (40 μl for group 1; 25 μl for group 2) were taken at the designated time points from the retro-orbital sinus. Heparinized micropipettes were inserted into the sinus until full, then placed in Vacutainer tubes with enough autoclaved, distilled, deionized water to yield 1 ml total solution. Samples were frozen until ethanol levels could be determined; after thawing, the solutions were incubated at 60°C for 15 min and the head space gas in the tubes was subjected to gas chromatography. Ethanol concentrations (determined by comparison with standards) were plotted against hours after withdrawal; the slope of this line was the calculated elimination rate (in mg% × hr^{-1}); the y-intercept gave the ethanol concentration at time zero (C_0).

TABLE 1

SUMMARY OF EFFECTS OF ETHANOL TREATMENT AND GENDER ON SELECTED BEHAVIORAL AND PHYSIOLOGICAL VARIABLES

Variable	Control		Ethanol treated		Significance levels		
	Females	Males	Females	Males	Treatment	Gender	Interaction
Seizure score	0.10 ± 0.10	0.00 ± 0.00	0.70 ± 0.26	1.00 ± 0.21	‡	N.S.	N.S.
Rectal temperature, °C	38.26 ± 0.13	38.18 ± 0.11	37.34 ± 0.36	35.76 ± 0.58	‡	*	*
Hole-in-wall crossings	4.40 ± 1.58	12.40 ± 2.32	2.20 ± 1.10	1.30 ± 0.54	‡	*	†
Hole-in-wall vicarious entries	15.60 ± 1.54	12.50 ± 2.58	8.10 ± 1.77	4.20 ± 0.71	‡	N.S.	N.S.
Hole-in-wall rearing	5.80 ± 1.56	10.30 ± 1.51	1.40 ± 0.70	1.90 ± 0.71	‡	?	N.S.
Vertical screen crossings	47.70 ± 3.93	57.20 ± 4.26	35.20 ± 9.52	19.00 ± 3.95	‡	N.S.	*

Data expressed as mean ± SEM.

No significant differences were observed for treatment, gender or interaction for all variables not listed, except for vertical screen approaches to bottom (treatment difference, $p<0.05$) and vertical screen approaches to left side (treatment difference, $p<0.05$).

Significance levels are indicated as follows: *$p<0.05$; †$p<0.01$; ‡$p<0.001$; ? indicates borderline values ($p=0.05 \pm 0.005$).

RESULTS

Table 1 provides an overview of mean scores and significance levels for the relevant test variables. As expected, seizure scores were significantly different for the two treatment groups, $F(1,36)=20.95$, $p<0.001$. No gender effect or interaction between gender and group was found for this measure (gender: $F(1,36)=0.33$; interaction: $F(1,36)=1.31$). One female mouse in the control group exhibited a mild (score=1) seizure.

Body temperatures, however, showed not only a significant group difference, $F(1,36)=22.74$, $p<0.001$, but also that females were significantly less affected by withdrawal, $F(1,36)=5.65$, $p<0.05$, and that there was a significant interaction between gender and treatment, $F(1.36)=4.60$, $p<0.05$.

The results for hole-in-wall crossings were similar to those for body temperature. The measures were significantly different for the two treatment groups, $F(1,36)=18.81$, $p<0.001$, and a gender effect, $F(1,36)=5.36$, $p<0.05$, was observed: Ethanol-treated animals scored fewer crossings, and females of the ethanol group were less affected than males. An interaction between gender and group was also observed, $F(1,36)=8.42$, $p<0.01$: Male control animals had much higher scores than females, whereas there was no significant difference between males and females in the ethanol-treated group.

Hole-in-wall vicarious trial entry scores showed only a significant treatment effect, $F(1,36)=19.68$, $p<0.001$. A treatment effect was also seen for hole-in-wall rearing, $F(1,36)=27.98$, $p<0.001$. For this measure, females were less affected by ethanol treatment than were males, $F(1,36)=4.27$, $p<0.05$, although this gender effect showed only borderline significance.

Vertical screen crossings proved effective for differentiating between the ethanol-treated and control groups, $F(1,36)=18.38$, $p<0.001$. Although no gender effect was seen, there was a significant interaction between gender and group, as females were again less affected than males within the ethanol-treated group, $F(1,36)=4.72$, $p<0.05$.

Of the other measured variables, only vertical screen approaches to the bottom and approaches to the left side significantly differentiated the groups. No significant differences were found for any of the other measures in the first experiment.

The average body weight dropped from 27.7 to 22.3 g for ethanol-treated mice and from 27.8 to 22.1 g for control-group mice in the first experiment; this represents a loss of about 20% of the free-feeding weight for both groups. A two-tailed t-test revealed no significant difference in weight loss between groups, $t=0.53$, $p=0.60$. Across-group comparisons of weight loss (as percentage of free-feeding weight) for males vs females showed no significant differences (for females, the mean dropped from 25.4 to 20.8 g, or 18% loss; for males, from 29.9 to 23.7 g, or 21%; in both cases, $p>0.50$).

Paradoxically, males in the ethanol-treated group consumed significantly less diet (expressed as g ethanol/kg body weight) than females. Consumption for males (mean±SEM) was 134.0±4.8 g/kg, while females consumed 156.6±6.4 g/kg during the 9-day period. This difference was significant, $t=-2.80$, $p<0.05$.

Since amount of diet consumed did not appear to explain the gender differences and interactions observed, we calculated Pearson correlations of scores versus order in which animals were tested for both males and females in the ethanol-treated group. The first animal tested in each group was observed 6 hr post-withdrawal, while the last (10th) of each group was tested about 7 hr post-withdrawal. Although there were no significant correlations for females, males showed significant correlations between order of testing and the seizure score, $r=-0.61$, $p<0.05$, body temperature, $r=0.84$, $p<0.01$, and vertical screen crossing measures, $r=0.80$, $p<0.01$, although not for hole-in-wall crossings, $r=0.15$, hole-in-wall vicarious trial entries, $r=0.45$, or hole-in-wall rearings, $r=0.04$.

In the second experiment, which measured rate of ethanol clearance from the blood, no gender differences were found in a one-way analysis of variance for rates (mean ± SEM for females, 95.0±8.3 mg% × hr^{-1}; for males, 93.1±10.6 mg% × hr^{-1}). A test for homogeneity of variance supported the validity of analyzing the groups by this method.

A concentration effect on rate of ethanol disappearance from the blood was also found. There was a significant correlation between C_0 and the rate of ethanol elimination, $r=0.84$, $p<0.01$.

DISCUSSION

Of the 21 measures that we employed, 6 tests (seizure score; body temperature; hole-in-wall crossings, trial entries and rearing; vertical screen crossings) proved highly effective in differentiating ethanol-dependent from control animals. If is our belief that these measures would be quite useful in identifying high- and low-ethanol-dependence mice for selection studies or other ethanol research involving genetically heterogenous mice.

We found that female ethanol-treated mice consume significantly more ethanol than do males on a g/kg basis. Nevertheless, they show a less severe withdrawal syndrome as indicated by all three of the measures for which gender differences existed, as well as on all three variables for which an interaction was found. Few other animal studies have examined gender differences in degree of ethanol dependence (as measured by the effects of ethanol withdrawal), since most have used males only. Nevertheless, differences attributable to gender were apparent in those studies that did examine withdrawal symptoms of both male and female rodents.

For example, Goldstein found that the pooled seizure scores of female mice were about half those of males for both F_1 and F_2 generations of her 1973 selection study [11]; she noted that these differences were attributable to lower blood alcohol levels in females at the time of testing. Collins *et al.* [3] found that C57BL mice 10 to 25 days older than those used in this study exhibit gender differences in rate of ethanol elimination in a single-dose test: Females eliminated ethanol almost twice as fast as males. (They did not find sex differences, however, in younger animals.) They also reported a similar gender difference for BALB mice. On the other hand, we found no gender difference in elimination rate at 85 days of age. There are two obvious differences between the studies which may account for the dissimilar data: first, the use of heterogeneous (HS) rather than homogeneous (inbred) stock; second, chronic administration of ethanol vs administration of a single dose. Attempts are now in progress to determine if these two treatments have different effects on ethanol elimination rate within and/or between sexes. It should be noted that C57BL and BALB are two of the eight parent strains of our HS stock [4].

We also found that female HS mice exhibited greater acceptance of ethanol than did males. Other studies have described gender differences in ethanol preference. For example, Goodrick found a higher preference for ethanol in female hybrid mice, but noted that most of the sex difference was accounted for by the C57BL parentage of the females [14]. C57BL female mice, but not CBA, were shown to exhibit greater ethanol preference than males in another study [5]. The effect of chronic intermittent administration of ethanol on an avoidance task was less in female than in male rats [31]. As noted earlier, few other rodent studies have examined gender differences, either for withdrawal symptoms or for effects of chronic administration.

Thus, the mechanism behind the gender difference observed in the present study may be a differential effect of ethanol withdrawal that is not due to elimination rate disparities, but rather arises from other gender-specific differences. Ethanol has been shown to have severe effects on the hypothalamic-pituitary-gonadal axis in male rats [2]. Only further study will elucidate all the mechanisms involved.

In addition, as noted by Reed and Kalant [27], differences in adiposity may affect the apparent rate of ethanol metabolism and the subject's overall reaction to ethanol. More adipose tissue (relative to total body mass) would result in a higher dose of ethanol per gram of lean body mass. It is our assumption that these factors are minimal in the present experiment; the loss of approximately 20% of the free-feeding weight no doubt reduces the amount of adipose tissue present and relative differences in adiposity (e.g., between sexes) would be minimized.

Finally, the apparent effect of C_0 on rate of ethanol elimination from the blood is in some ways similar to that reported by Wendell and Thurman [33]. However, in their study, concentration effects were eliminated in ethanol-treated rats. Our findings may differ due to the methodological differences, primarily the amount of time subjects are fed an ethanol diet (9 days vs 3 to 5 weeks) and the methods of blood sampling (retro-orbital sinus vs tail vein), or to species differences.

In any case, the results presented here indicate that the practice of using only males for ethanol-dependence tests may be ill-advised. Although the exclusion of female animals has been justified by the need to eliminate possible effects of the estrous cycle, it may be that important gender differences have not been studied sufficiently.

It is our hope that the multifaceted procedure reported in this paper will lead to development of a reliable, consistent battery of measures for differentiating high- and low-dependence mice in order to breed selectively for differences in alcohol dependence without the confounding effects of elimination rate. If these tests can help elucidate the genetic component of alcohol dependence in mice, further studies of the role of inheritance in human alcoholism may be indicated.

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