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## Bidirectional Selection for Susceptibility to Ethanol Withdrawal Seizures in *Mus musculus*

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*Using within-family selection from a genetically heterogeneous population of HSI/bg mice, lines and replicates have been selected for high (withdrawal seizure prone; WSP) and low (withdrawal seizure resistant; WSR) susceptibility to convulsions after withdrawal from chronic exposure to ethanol. Two nonselected control lines (withdrawal-seizure control; WSC) have also been maintained. The response was bidirectional in both replicates across 11 selected generations, WSP and WSR lines differing approximately 10-fold in seizure severity after an identical regimen of chronic exposure to ethanol. Realized heritability was found to be approximately 0.28. The phenotype appears to be polygenic in nature. The relatively low amount of inbreeding in these lines and the large response to selection should make them useful for examining the physiological basis of physical dependence on ethanol.*

**KEY WORDS:** pharmacogenetics; mice; selective breeding; alcoholism; ethanol physical dependence; ethanol withdrawal; withdrawal seizure-prone (WSP) and withdrawal seizure-resistant (WSR) lines; handling-induced convulsions.

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## INTRODUCTION

A preliminary report by Goldstein (1973) suggested that it might be possible to breed mice selectively for ethanol withdrawal convulsion severity. Withdrawal convulsions are a symptom of ethanol withdrawal common to all species studied (Kalant, 1977; Friedman, 1980). Furthermore, they are easily quantified and show dose- and duration-response relations for ethanol treatment doses (Goldstein, 1972). While the role of physical dependence on alcohol in the development and maintenance of alcoholism is unclear (Mello, 1972; Turner, 1980), an illuminating discussion of the relevant issues is given by Cappell and LeBlanc (1979), who conclude that animal models of alcoholism need not necessarily include a state of physical dependence as a criterion; yet the study of physical dependence in animals remains critical to our understanding of alcohol's pathological actions. We had previously reported that mice rendered physically dependent on ethanol by force exposure to ethanol vapor became physically dependent and subsequently exhibited voluntary exposure to ethanol vapor in preference to air (Rijk *et al.*, 1982). We chose, therefore, to breed mouse lines selectively for the severity of withdrawal seizures after 3 days of forced inhalation.

We had two goals. First, we wanted to develop lines that would provide a valuable resource for mechanism-directed experiments designed to explicate those actions of ethanol on the central nervous system critical for the development of ethanol physical dependence and withdrawal. Second, we were interested in estimating the relative genetic contribution to the phenotype of ethanol withdrawal and in identifying correlated response to selection. In a preliminary report, we documented the response to selection after five generations (Crabbe *et al.*, 1983a). In this paper, we report the result of 11 generations of selection and describe some genetic characteristics of the project.

## MATERIALS AND METHODS

*Foundation Stock.* Twenty pairs of HS/Ibg mice from Generation 29 were purchased from the Institute for Behavioral Genetics, Boulder, Colorado. Eighteen matings of Generation 30 parents excluding common grandparents were divided at random into two groups of nine families each. These Generation 31 HS mice became the foundation ( $S_0$ ) population for selection. All offspring from first matings were made physically dependent on ethanol and were then tested for ethanol withdrawal severity as described below.

*Establishment of Lines.* One group of nine families was designated the first replicate of the experiment, and the other served as the second replicate. Within-family selection was generally followed in establishing the selected lines (Falconer, 1960). First, one female and one male mouse

from each family tested were chosen at random to form the withdrawal-seizure control (WSC-1) line. Second, of the remaining mice, the highest-scoring male and female from each family were chosen to form the withdrawal seizure-prone (WSP-1) line. Finally, the lowest-scoring male and female from each family were chosen to form the withdrawal seizure-resistant (WSR-1) line. These mice were Generation  $S_0$ . Matings were established according to a rotational system suggested by Dr. Carl Hansen (NIH) such that the female from Family 1 was always mated to the male from Family 2, the female from Family 2 to the male from Family 3, and so forth. Additional matings were used to establish two to six reserve families for each line in order to replace missing litters or provide animals in cases of single-sex litters. One week later, this process was repeated in the other set of nine families to form the reproductively isolated second replicate.

*Ethanol Physical Dependence and Withdrawal.* The model for induction of physical dependence on ethanol has been published (Crabbe *et al.*, 1983b). Between 0800 and 0900 h, mice are weighed and injected with 1.5 g/kg ethanol (20% v/v, ip) to induce initial ethanol concentrations. Each mouse is also injected with pyrazole HCl (1 mmol/kg, ip) to inhibit alcohol dehydrogenase (ADH). All mice are then placed in hanging wire-mesh cages suspended inside large chambers through which ethanol vapor (5–15 mg/liter air) is continuously pumped. After 24 and 48 h of vapor exposure, mice are temporarily removed from the chamber, weighed, and reinjected with pyrazole, and the ethanol vapor concentration is raised. After 72 h of vapor inhalation (24 h after the last pyrazole injection) all mice are withdrawn from ethanol vapor and weighed, a blood sample is drawn from the tail for determination of blood ethanol concentrations (BEC) at the time of withdrawal, and mice are scored for ethanol withdrawal convulsions each hour for 15 h and again at h 24 and 25. All scoring for the mice reported here was performed by A. Kosobud, who was unaware of the line of the mouse at the time of scoring (Table I).

The results of breeding permit approximately 85% of the mice to be tested at  $42 \pm 3$  days and 15% at  $37 \pm 1$  days. After testing, mice are allowed to recover for 3 weeks before selected mice are mated (thus, at 8–9 weeks old). Except as noted, in Generation  $S_0$ , the control lines are never tested for physical dependence on ethanol.

*Selection Index.* The severity of withdrawal is assessed by the handling-induced convulsion (Goldstein, 1972). Scores are assigned according to the scale described in Table I. Each mouse's hourly withdrawal scores are collected and the area under the 25 h withdrawal curve is calculated. The 15-h area is also calculated, as well as the peak withdrawal score (average of the highest score plus the score 1 h before and 1 h after the highest score). Selection is made on the basis of the 25-h area.

**Table I.** Handling-Induced Convulsion Scoring Scale

Score	Description
4	Severe tonic-clonic convulsion when lifted by the tail, with immediate onset and long duration, often continuing for several seconds after the mouse is released
3	Tonic-clonic convulsion when lifted by the tail, often with onset delayed by as much as 1-2 s
2	Tonic convulsion when lifted by the tail or tonic-clonic convulsion following gentle spin through a 180° arc
1	No convulsion when lifted by the tail, but tonic convulsion elicited by spin
0.5	Only facial grimace following spin
0	No convulsion

*Procedure Followed for Selection Study.* In Generations S<sub>0</sub>-S<sub>2</sub>, all mice of one sex in each replicate (thus, the WSP-1 and WSR-1 females, for example) were tested in a single inhalation chamber. Therefore, for Generations S<sub>1</sub> and S<sub>2</sub>, a direct ascertainment of the response to selection was available within gender and replication by comparing the withdrawal scores since these mice received identical ethanol exposure. However, the line differences (WSP versus WSR) became more pronounced than the sex differences, and from Generation S<sub>3</sub> on it was necessary to administer more ethanol to WSR mice than to WSP mice in order to produce physical dependence in the WSR mice without killing the WSP mice. Thus, from S<sub>3</sub> onward all female and male WSP mice from one replicate were tested in one chamber, and the WSR mice were tested in a separate chamber (see Table II). Occasionally (Generations S<sub>2</sub>, S<sub>5</sub>, and S<sub>6</sub>), insufficient litters were available at the time of testing to ensure acceptably low levels of inbreeding. In such instances, a third week of withdrawal seizure induction and testing was performed.

*Estimates of the Response to Selection.* From S<sub>3</sub> onward, in order to estimate the response to selection it was necessary to test additional WSP and WSR mice, from second litters bred specifically for that purpose, in the same inhalation chamber and compare subsequent withdrawal. Different groups of mice were tested together in different generations. The specific groupings are given in Table II. During the 72-h period of exposure, we attempted to maintain ethanol vapor concentrations in the two chambers as nearly equal as possible. For example, the average chamber concentrations (mg EtOH/liter air) for each day of exposure in S<sub>11</sub> were 6.8, 9.9, and 12.3 for the females and 6.5, 9.5 and 12.0 for the males. However, withdrawal scores for any group for the experiments designed to estimate the response to selection are strictly com-

**Table II.** Lines and Sexes of Mice Tested Together in the Same Inhalation Chamber for Standard Selection Procedure and for Additional Experiments Carried Out to Test Response to Selection

Generation(s)	Week 1		Week 2	
	Chamber 1	Chamber 2	Chamber 1	Chamber 2
Standard selection procedure				
S <sub>0</sub> -S <sub>2</sub>	P1 + R1 Females	P1 + R1 Males	P2 + R2 Females	P2 + R2 Males
S <sub>3</sub> and thereafter	P1 Females + males	R1 Females + males	P2 Females + males	R2 Females + males
Groups specifically bred to estimate response to selection <sup>a</sup>				
S <sub>0</sub> -S <sub>2</sub>	Groups available from standard selection procedure (see above)			
S <sub>3</sub>	P1 + R1 + C1 Females + males	P2 + R2 + C2 Females + males	—	—
S <sub>5</sub>	P1 + R1 + C1 Females	P1 + R1 + C1 Males	P2 + R2 + C2 Females	P2 + R2 + C2 Males
S <sub>7</sub>	P1 + R1 + P2 + R2 Females + males	—	—	—
S <sub>10</sub> , S <sub>11</sub>	P1 + R1 + C1 Females	P1 + R1 + C1 P2 + R2 + C2 Males	—	—

<sup>a</sup> Within a chamber and week, ethanol exposure was identical for all animals. When testing was performed during two successive weeks, exposure conditions in Chamber 1 cannot, therefore, be considered to be equal in the two weeks. As noted in the text, within a week, Chamber 1 and Chamber 2 ethanol levels were generally quite close for groups specifically bred to estimate response to selection. (P1 = WSP1; R1 = WSR1, etc.)

parable only for other groups of mice treated in the same chamber in the same week.

## RESULTS

*Response to Selection.* In Table III, the mean ± SE for each sex and line for the area under the withdrawal curve (selection index), the peak withdrawal score, and the BEC at the time of withdrawal are shown for each generation. It should be noted that the comparisons across lines, replicates, and generations are not generally valid (see Table III, footnote a) since animals were tested in different chambers. These data represent all animals tested (WSC mice were not tested).

As noted, direct comparisons were made with mice from Generations S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub>, S<sub>5</sub>, S<sub>7</sub>, S<sub>10</sub>, and S<sub>11</sub> (see Materials and Methods) to estimate

Table III. Ethanol Withdrawal-Seizure Selection Score, Peak Ethanol Withdrawal Score, and Blood Ethanol Concentration (BEC) for All Mice Tested Each Generation<sup>a</sup>

Generations of selection	Area 25			Peak			BEC (mg/ml)			
	P <sub>1</sub>	R <sub>1</sub>	P <sub>2</sub>	R <sub>1</sub>	P <sub>2</sub>	R <sub>2</sub>	P <sub>1</sub>	R <sub>1</sub>	P <sub>2</sub>	R <sub>2</sub>
Female										
S <sub>0</sub>	19.9 ± 2.9	19.9 ± 2.9	24.8 ± 2.8	2.07 ± 0.21	1.00 ± 0.17	1.00 ± 0.17	3.33 ± 0.17	3.33 ± 0.17	3.51 ± 0.19	3.51 ± 0.19
S <sub>1</sub>	36.4 ± 2.6	28.1 ± 3.3	56.3 ± 2.5	2.96 ± 0.17	2.29 ± 0.19	2.89 ± 0.14	3.10 ± 0.17	3.10 ± 0.14	2.68 ± 0.14	2.70 ± 0.13
S <sub>2</sub>	20.1 ± 3.2	12.1 ± 2.0	15.2 ± 2.5	1.91 ± 0.23	1.37 ± 0.15	1.77 ± 0.19	0.81 ± 0.15	0.81 ± 0.15	1.88 ± 0.12	2.12 ± 0.29
S <sub>3</sub>	11.7 ± 1.7	8.6 ± 1.6	29.1 ± 4.0	1.37 ± 0.14	1.01 ± 0.14	2.29 ± 0.23	1.45 ± 0.15	1.45 ± 0.15	—	—
S <sub>4</sub>	10.7 ± 1.4	5.4 ± 0.9	20.7 ± 2.3	1.20 ± 0.12	0.82 ± 0.12	2.28 ± 0.17	1.17 ± 0.25	1.17 ± 0.25	0.61 ± 0.06	0.76 ± 0.07
S <sub>5</sub>	7.1 ± 1.3	5.4 ± 1.1	32.7 ± 3.5	0.77 ± 0.10	0.75 ± 0.10	2.06 ± 0.15	0.84 ± 0.13	0.84 ± 0.13	0.37 ± 0.04	1.62 ± 0.11
S <sub>6</sub>	11.8 ± 1.7	12.3 ± 2.0	27.4 ± 3.1	1.10 ± 0.12	1.13 ± 0.11	1.19 ± 0.16	0.80 ± 0.12	0.80 ± 0.12	0.26 ± 0.04	2.59 ± 0.11
S <sub>7</sub>	30.5 ± 2.1	9.0 ± 1.5	43.0 ± 2.5	2.51 ± 0.13	1.21 ± 0.13	2.90 ± 0.13	1.66 ± 0.18	1.66 ± 0.18	1.38 ± 0.09	2.11 ± 0.09
S <sub>8</sub>	33.6 ± 3.5	4.1 ± 1.1	57.9 ± 4.1	2.25 ± 0.13	0.58 ± 0.12	3.50 ± 0.15	1.43 ± 0.13	1.43 ± 0.13	1.80 ± 0.10	2.25 ± 0.08
S <sub>9</sub>	36.1 ± 2.6	3.8 ± 1.3	41.2 ± 3.0	2.02 ± 0.13	0.57 ± 0.12	2.93 ± 0.11	0.98 ± 0.17	0.98 ± 0.17	0.64 ± 0.07	1.34 ± 0.08
S <sub>10</sub>	28.4 ± 2.0	2.2 ± 0.6	62.2 ± 2.8	2.01 ± 0.14	0.37 ± 0.08	3.58 ± 0.12	1.30 ± 0.11	1.30 ± 0.11	1.63 ± 0.12	2.51 ± 0.11
S <sub>11</sub>	37.0 ± 2.0	3.8 ± 1.2	53.1 ± 2.8	2.51 ± 0.10	0.51 ± 0.10	3.47 ± 0.09	1.21 ± 0.15	1.21 ± 0.15	2.76 ± 0.08	3.83 ± 0.17
Male										
S <sub>0</sub>	9.1 ± 2.2	9.1 ± 2.2	12.8 ± 1.9	0.94 ± 0.15	0.94 ± 0.15	0.84 ± 0.14	0.84 ± 0.14	0.84 ± 0.14	2.17 ± 0.11	2.17 ± 0.11
S <sub>1</sub>	20.9 ± 2.9	18.6 ± 3.9	53.5 ± 2.8	1.88 ± 0.17	1.85 ± 0.25	3.00 ± 0.14	2.30 ± 0.10	2.30 ± 0.10	4.76 ± 0.50	4.71 ± 0.27
S <sub>2</sub>	16.9 ± 2.0	9.0 ± 2.4	19.8 ± 3.3	1.85 ± 0.17	1.12 ± 0.17	1.88 ± 0.23	0.80 ± 0.18	0.80 ± 0.18	2.51 ± 0.10	2.27 ± 0.11
S <sub>3</sub>	10.1 ± 2.0	20.0 ± 2.7	19.5 ± 2.4	1.11 ± 0.13	1.62 ± 0.20	2.02 ± 0.17	1.86 ± 0.18	1.86 ± 0.18	—	—
S <sub>4</sub>	12.3 ± 1.6	8.3 ± 1.2	19.7 ± 2.7	1.24 ± 0.09	1.03 ± 0.10	1.99 ± 0.19	0.97 ± 0.10	0.97 ± 0.10	0.43 ± 0.04	1.19 ± 0.08
S <sub>5</sub>	6.1 ± 1.3	9.7 ± 1.8	34.2 ± 4.7	0.65 ± 0.09	0.88 ± 0.12	2.28 ± 0.17	1.16 ± 0.16	1.16 ± 0.16	0.27 ± 0.04	1.63 ± 0.11
S <sub>6</sub>	9.7 ± 1.7	18.6 ± 1.8	27.4 ± 3.1	1.00 ± 0.12	1.62 ± 0.11	2.24 ± 0.20	1.88 ± 0.15	1.88 ± 0.15	0.20 ± 0.05	2.44 ± 0.10
S <sub>7</sub>	37.2 ± 2.7	8.4 ± 1.3	50.3 ± 2.5	2.51 ± 0.13	1.30 ± 0.14	3.00 ± 0.14	1.88 ± 0.15	1.88 ± 0.15	1.54 ± 0.06	2.04 ± 0.07
S <sub>8</sub>	34.4 ± 3.5	7.0 ± 1.6	64.0 ± 2.8	2.30 ± 0.17	1.00 ± 0.15	3.77 ± 0.10	1.96 ± 0.17	1.96 ± 0.17	1.83 ± 0.09	2.08 ± 0.08
S <sub>9</sub>	36.1 ± 2.6	5.8 ± 1.3	47.4 ± 3.2	2.42 ± 0.12	0.96 ± 0.14	3.19 ± 0.14	1.02 ± 0.14	1.02 ± 0.14	2.09 ± 0.17	2.26 ± 0.08
S <sub>10</sub>	37.0 ± 3.4	3.5 ± 0.7	59.2 ± 3.7	2.45 ± 0.15	0.49 ± 0.08	3.40 ± 0.15	1.32 ± 0.14	1.32 ± 0.14	2.61 ± 0.12	2.03 ± 0.16
S <sub>11</sub>	46.0 ± 3.1	5.4 ± 1.2	59.3 ± 2.9	2.98 ± 0.13	0.77 ± 0.12	3.56 ± 0.09	1.20 ± 0.13	1.20 ± 0.13	3.39 ± 0.14	1.57 ± 0.08

<sup>a</sup> Means ± SE are shown for all mice tested each generation. Comparisons across generations, lines, and replications are not generally possible, since animals were tested in different inhalation chambers. Values for S<sub>0</sub> do not differ across lines because mice had not yet been assigned to lines. For S<sub>1</sub> and S<sub>2</sub>, comparisons can be made between lines within sex, generation, and replicate (see text). From S<sub>3</sub> onward direct comparisons are restricted to those between gender within line, replication, and generation (see text and top of Table II). BEC samples from S<sub>3</sub> were inadvertently destroyed. Number of mice tested for each group is given in Table V.

the response to selection. Seizure scores during ethanol withdrawal are given in Table IV. Within sex and replication, the prone lines clearly display high withdrawal-convulsion scores and the resistant lines low scores, with the control lines intermediate. The WSC-2 line has had relatively low withdrawal scores, apparently since Generation 3. Statistics supporting these generalizations are given in Table IV.

*Role of Ethanol Metabolism.* It is possible that the WSP and WSR lines could have developed differential metabolic responses to ethanol as a correlated response to selection. Since severity of withdrawal is a joint function of duration and concentration of ethanol vapor exposure in an inhalation system such as ours (Goldstein, 1972), we routinely monitor the blood ethanol concentration (BEC) at the time of withdrawal by analyzing a 20- $\mu$ l blood sample taken from the tip of the tail (Crabbe *et al.*, 1983b). The mean BECs for all mice tested for selection are given in Table III, while those for each of the direct WSP vs. WSR comparisons are given in Table IV. Statistical comparisons are shown in Table IV.

Considering the within-sex comparisons between WSP-1 and WSR-1 mice in Table IV, no difference was apparent during early generations of selection (through S<sub>7</sub>). By S<sub>10</sub> and S<sub>11</sub>, it appears that WSP-1 mice achieve BECs approximately 30% higher than those of the corresponding WSR-1 mice, at least in males. WSC-1 females closely resemble the WSP-1 mice, while male WSC-1 mice seem generally to achieve BECs intermediate to those of the selected lines. Thus, the large difference between WSP-1 and WSR-1 mice in withdrawal severity is to some degree possibly due to their achieving different tissue concentrations of ethanol, at least in males. The WSP-2 and WSR-2 mice also did not appear to differ systematically on BEC in early generations. Female and male WSP-2 mice reached higher BECs than female and male WSR-2 mice in S<sub>10</sub> (26 and 69%, respectively), but no differences were seen in S<sub>11</sub>. Thus, it does not seem likely that the WSP-2 versus WSR-2 differences in withdrawal severity can be attributed to different doses of ethanol. WSC-2 female mice have consistently reached lower BECs than either selected line, a finding for which we have no explanation. This trait was also exhibited by male WSC-2 mice in Generations S<sub>3</sub> and S<sub>11</sub>, although in other generations they resembled WSR-2 mice. The lower BECs attained in the WSC-2 line could explain why they exhibit generally low withdrawal scores. In other studies (Kosobud *et al.*, 1984), we have found that the prone and resistant lines metabolize ethanol during the withdrawal period at equivalent rates.

*Estimates of Heritability.* Inspection of Tables III and IV reveals that the raw seizure scores vary considerably from generation to generation. This occurs at least in part because of the variability in ethanol

Table IV. Direct Comparisons of Ethanol Withdrawal-Seizure

Generations of selection	WSP1		WSC1		WSR1	
	Area 25	BEC	Area 25	BEC	Area 25	BEC
Female						
S <sub>1</sub>	36.4 ± 2.6	3.10 ± 0.17	—	—	28.1 ± 3.3	3.10 ± 0.14
S <sub>2</sub>	20.1 ± 3.3	2.63 ± 0.08	—	—	12.1 ± 2.0*	2.37 ± 0.07*
S <sub>3</sub>	11.3 ± 2.6	0.97 ± 0.08	7.4 ± 1.7	0.99 ± 0.12	3.4 ± 0.9*	0.88 ± 0.15
S <sub>5</sub>	38.5 ± 4.4	1.50 ± 0.10	22.0 ± 4.6***††	1.71 ± 0.11	9.3 ± 1.8***	1.53 ± 0.10
S <sub>7</sub>	26.7 ± 4.1	0.91 ± 0.29	—	—	3.1 ± 1.9***	1.26 ± 0.22
S <sub>10</sub>	41.4 ± 3.5	3.11 ± 0.14	30.2 ± 6.2†††	3.04 ± 0.16†††	1.1 ± 0.4***	2.34 ± 0.07***
S <sub>11</sub>	46.9 ± 3.8	3.66 ± 0.33	34.6 ± 4.1*†††	3.78 ± 0.28†	3.4 ± 1.1***	2.92 ± 0.17
Male						
S <sub>1</sub>	20.9 ± 2.9	4.76 ± 0.50	—	—	18.6 ± 3.9	4.71 ± 0.27
S <sub>2</sub>	16.9 ± 2.0	2.51 ± 0.10	—	—	9.0 ± 2.4*	2.27 ± 0.11
S <sub>3</sub>	6.7 ± 1.6	0.63 ± 0.13	6.9 ± 1.5	0.56 ± 0.13	8.1 ± 3.3	0.77 ± 0.31
S <sub>5</sub>	49.7 ± 4.5	2.05 ± 0.15	26.2 ± 4.1***	1.73 ± 0.12	22.8 ± 3.7***	1.56 ± 0.12**
S <sub>7</sub>	36.3 ± 1.7	1.64 ± 0.61	—	—	7.9 ± 4.6**	1.57 ± 0.32
S <sub>10</sub>	43.3 ± 4.7	2.80 ± 0.19	24.0 ± 4.4***†††	2.17 ± 0.16*	5.1 ± 1.3***	2.03 ± 0.18*
S <sub>11</sub>	46.7 ± 3.9	4.03 ± 0.24	38.9 ± 3.7†††	3.23 ± 0.19*†††	4.4 ± 2.7***	2.29 ± 0.10***

\* The area under the ethanol withdrawal handling-induced convulsion curve in score-hours is shown. BEC, blood ethanol concentration (mg/ml) at the time of withdrawal. All values are means ± SE. All mice from Generations S<sub>3</sub> to S<sub>11</sub> whose data are shown here were from second or subsequent litters and thus were not part of the selection experiment per se. To determine which sexes, lines, and replicates were exposed to identical ethanol vapor concentrations, refer to Table II. Control lines were not tested for Generation S<sub>1</sub>, S<sub>2</sub>, or S<sub>7</sub>.

vapor concentrations over a 72-h exposure. Since environmental conditions were not invariant across generations, we estimated the realized heritability in the WSP/WSR lines from normalized response differences. Each generation, the response of each mouse tested (within line, sex, and replicate, for mice shown in Table III) was converted to a Z score derived from the mean and standard deviation of its group. The average Z score for the mice contributing offspring to the next generation was taken to be the selection differential, shown as *S* in Table V. Since selection was within family, this procedure essentially corrected for the different exposure conditions. The absolute value of *S* was averaged over sex and summed over line (thus, WSP-1 male and female plus WSR-1 male and female, divided by 2) to yield the overall selection differential per generation. These values were then cumulated over generations.

In order to estimate the response to selection (*R*) in a given generation, we exposed WSP and WSR mice to identical ethanol treatments (see Materials and Methods) and calculated their withdrawal scores as described. To estimate *R* within a replicate and generation, we then took the differences (prone minus resistant) in Area 25 shown in Table IV within each sex and normalized them to the estimated population phenotypic standard deviation (SD). To estimate the phenotypic SD, we employed a weighted estimate derived from the phenotypic SDs measured

Scores and Blood Ethanol Concentrations (BEC) for Selected Lines\*

WSP2		WSC2		WSR2	
Area 25	BEC	Area 25	BEC	Area 25	BEC
55.0 ± 3.0	2.68 ± 0.14	—	—	47.8 ± 1.9*	2.70 ± 0.41
15.2 ± 2.5	1.88 ± 0.12	—	—	5.5 ± 1.3***	2.12 ± 0.29
15.5 ± 3.5	1.46 ± 0.16	5.3 ± 1.6**	0.46 ± 0.13***†††	2.8 ± 1.0***	1.58 ± 0.05
37.9 ± 4.2	1.68 ± 0.10	14.3 ± 2.9***	1.49 ± 0.13	8.8 ± 2.0***	1.71 ± 0.13
35.2 ± 10.4	0.82 ± 0.13	—	—	6.2 ± 3.8*	0.95 ± 0.40
53.7 ± 4.3	3.15 ± 0.15	7.4 ± 2.7***	1.90 ± 0.15***††	2.1 ± 1.0***	2.50 ± 0.15**
61.9 ± 4.3	3.73 ± 0.30	8.8 ± 2.4***	2.53 ± 0.10***††	7.2 ± 1.5***	3.59 ± 0.25
53.5 ± 2.8	2.35 ± 0.11	—	—	38.7 ± 2.0***	1.98 ± 0.13*
19.8 ± 3.3	1.25 ± 0.16	—	—	5.1 ± 1.3***	1.34 ± 0.14
13.4 ± 1.9	1.25 ± 0.16	7.3 ± 3.4	0.23 ± 0.11***†††	7.5 ± 1.9*	0.99 ± 0.08
62.5 ± 5.1	1.79 ± 0.23	16.5 ± 2.6***	1.67 ± 0.11	11.2 ± 1.8***	1.81 ± 0.11
56.2 ± 8.7	1.72 ± 0.37	—	—	12.3 ± 3.3**	1.52 ± 0.41
53.1 ± 3.5	2.78 ± 0.20	10.5 ± 2.6***	1.53 ± 0.16***	6.6 ± 2.9***	1.64 ± 0.15***
71.4 ± 3.3	3.03 ± 0.17	12.2 ± 2.1***	2.35 ± 0.24*†	6.9 ± 1.8***	3.33 ± 0.32

\* *P* < 0.05 versus WSP line of same sex, generation, and replicate (two-tailed *t* test).

\*\* *P* < 0.01 versus WSP line of same sex, generation, and replicate (two-tailed *t* test).

\*\*\* *P* < 0.001 versus WSP line of same sex, generation, and replicate (two-tailed *t* test).

† *P* < 0.05 versus WSR line of same sex, generation, and replicate (two-tailed *t* test).

†† *P* < 0.01 versus WSR line of same sex, generation, and replicate (two-tailed *t* test).

††† *P* < 0.001 versus WSR line of same sex, generation, and replicate (two-tailed *t* test).

in the original S<sub>0</sub> populations and those measured in the WSC-1 and WSC-2 nonselected lines in Generation S<sub>11</sub> for each replicate. This value was 13.11 units (range of four estimates was 7.85–14.57 units).

Regressions of cumulated selection differential on response to selection, using data shown in Tables IV and V, are shown in Fig. 1. For the first replicate,  $r_{xy} = 0.956$  and the slope estimate of  $h^2$  was 0.231. In the second replicate, those values were  $r_{xy} = 0.956$  and  $h^2 = 0.299$ , yielding a combined estimate of  $h^2 = 0.265$ .

These values of  $h^2$  agree well with estimates derived from the total realized response to selection after 11 generations. Mice in S<sub>11</sub> were rendered physically dependent on ethanol and withdrawal was tested as described. All mice of one sex were treated in a single chamber, and those of the other sex in an identical chamber. As noted (see Materials and Methods), the differences in chamber ethanol concentrations were small. Estimates of realized heritability from the normalized total response to selection after 11 generations and the cumulated selection differential (through S<sub>10</sub>) were  $h^2 = 0.236$  in the first replicate and  $h^2 = 0.32$  in the second replicate. The combined estimate of realized heritability in the two replicates of the experiment is thus  $h^2 = 0.278$ . Our preliminary report of response to selection, based on S<sub>5</sub> data, overestimated  $h^2$  (Crabbe *et al.*, 1983a). Estimates from S<sub>7</sub>, S<sub>10</sub>, and S<sub>11</sub>, calculated from

Table V. Genetic Data for Ethanol Withdrawal Seizure-Prone (WSP), Withdrawal Seizure-Resistant (WSR), and Withdrawal-Seizure Control (WSC) Lines<sup>a</sup>

Genera- tions of selection	WSP-1					WSP-2					WSP-3											
	Total tested	No. families	S	N	$\bar{X}(\sigma^2)$	Total tested	No. families	S	N	$\bar{X}(\sigma^2)$	Total tested	No. families	S	N	$\bar{X}(\sigma^2)$	Total tested	No. families	S	N	$\bar{X}(\sigma^2)$	Cum $\Delta F$	$N_e$
S <sub>0</sub>	62	9	0.598	18	2.00(22)	32.4	0.397	18	2.00(22)	32.4	—	—	—	—	—	66	10	0.713	18	1.80(16)	33.3	—
S <sub>1</sub>	59	9	0.899	18	2.00(22)	32.4	0.566	18	2.00(22)	32.4	0.0154	—	—	—	—	58	10	0.614	18	1.80(16)	33.3	0.0150
S <sub>2</sub>	80	10	0.675	16	1.60(24)	28.6	0.577	16	2.00(0.00)	34.0	0.0309	—	—	—	—	70	9	0.381	18	2.00(0.00)	36.0	0.0300
S <sub>3</sub>	65	10	0.994	18	1.80(16)	33.3	0.483	18	1.80(16)	33.3	0.0483	—	—	—	—	57	10	0.741	18	1.80(16)	33.3	0.0439
S <sub>4</sub>	78	9	0.649	18	2.00(0.00)	36.0	0.669	18	1.80(16)	33.3	0.0669	—	—	—	—	78	7	0.573	14	2.00(0.00)	28.6	0.0589
S <sub>5</sub>	65	8	0.907	16	2.00(0.00)	32.0	0.412	16	1.80(16)	33.3	0.0773	—	—	—	—	50	8	0.480	16	2.00(0.00)	32.0	0.0768
S <sub>6</sub>	75	8	0.824	14	1.80(19)	25.6	0.596	16	2.00(0.00)	32.0	0.0929	—	—	—	—	57	8	0.758	14	1.80(19)	25.6	0.0924
S <sub>7</sub>	66	10	0.434	18	1.80(16)	33.3	0.124	18	2.00(0.00)	32.0	0.124	—	—	—	—	69	5	1.074	10	2.00(0.00)	20.0	0.1119
S <sub>8</sub>	51	8	0.629	16	2.00(0.00)	32.0	0.1274	16	2.00(0.00)	32.0	0.1274	—	—	—	—	54	7	0.713	12	1.70(49)	19.3	0.1369
S <sub>9</sub>	71	8	0.372	16	2.00(0.00)	32.0	0.1430	16	2.00(0.00)	32.0	0.1430	—	—	—	—	63	6	1.022	12	2.00(33)	20.6	0.1629
S <sub>10</sub>	55	7	0.573	14	2.00(0.00)	28.0	0.1587	14	2.00(0.00)	28.0	0.1587	—	—	—	—	56	5	0.672	10	2.00(0.00)	20.0	0.1871
S <sub>11</sub>	63	9	0.750	16	1.80(17)	29.5	0.1765	18	1.80(16)	33.3	0.1765	—	—	—	—	57	7	0.677	14	2.00(29)	24.5	0.2121

Genera- tions of selection	WSC-1					WSC-2					WSC-3												
	Total tested	No. families	S	N	$\bar{X}(\sigma^2)$	Total tested	No. families	S	N	$\bar{X}(\sigma^2)$	Total tested	No. families	S	N	$\bar{X}(\sigma^2)$	Total tested	No. families	S	N	$\bar{X}(\sigma^2)$	Cum $\Delta F$	$N_e$	
S <sub>0</sub>	66	10	0.479	18	1.80(16)	33.3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>1</sub>	66	9	0.479	16	1.80(17)	29.5	0.0150	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>2</sub>	74	8	0.627	14	1.80(19)	25.4	0.0320	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>3</sub>	56	9	0.602	18	2.00(22)	32.4	0.0516	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>4</sub>	58	10	0.607	18	2.00(0.00)	36.0	0.0671	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>5</sub>	53	10	0.380	18	1.80(16)	33.3	0.0821	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>6</sub>	60	10	0.750	18	1.80(16)	33.3	0.0971	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>7</sub>	68	10	0.852	18	1.80(16)	33.3	0.1121	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>8</sub>	47	9	0.595	14	1.60(25)	24.9	0.1271	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>9</sub>	67	11	0.684	18	2.00(44)	29.5	0.1472	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>10</sub>	67	11	0.684	18	1.60(23)	32.3	0.1641	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S <sub>11</sub>	64	10	0.720	18	1.80(16)	33.3	0.1796	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

<sup>a</sup>Total number of mice tested is shown in the first column. Number of families, number of mice (N), and effective number of mice (N<sub>e</sub>) contributing tested offspring to the next generation are shown. Mean and variance [ $\bar{X}(\sigma^2)$ ] in family size and cumulated change in inbreeding coefficient ( $\Delta F$ ) were calculated as described in the text. Selection differentials (S) are the average Z score of the N chosen mice each generation, as described. WSC lines were not tested.

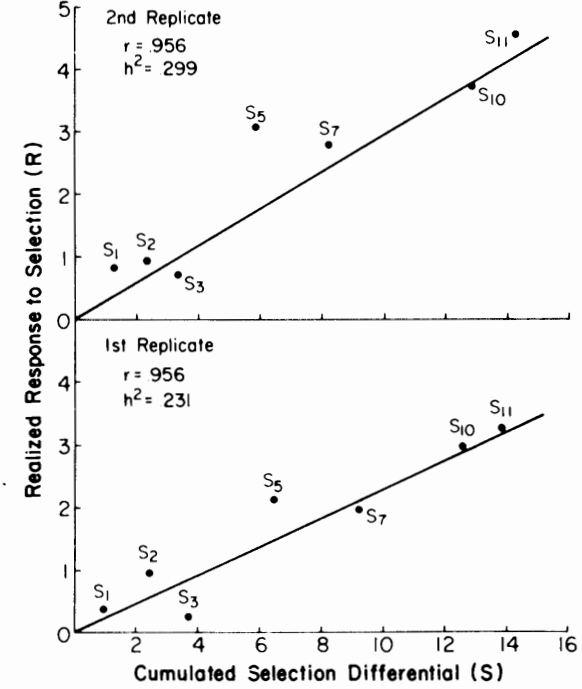


Fig. 1. Estimates of heritability for regression of normalized response to selection (R) on cumulated selection differential (S) for the data shown in Tables II–IV. Calculation of R and S is discussed in the text. First replicate, lower panel; second replicate, upper panel.

the total response to selection as just described for S<sub>11</sub>, were very stable (range in first replicate, 0.215–0.238; in second replicate, 0.292–0.339).

**Magnitude of Line Differences.** For the direct comparison of mice from S<sub>11</sub> just described, the 25-h area was analyzed with a three-factor ANOVA (Sex × Line × Replicate). The main effect of sex was not significant ( $F_{1,150} = 3.8, P > 0.05$ ), so we ignored the fact that the sexes were treated in different chambers. An example of the handling-induced convulsion scores during 25 h of withdrawal (for female mice from the first replicate) is shown in Fig. 2. The WSP-1 female mice had the most severe withdrawal convulsions, and the WSR-1 mice the least. WSC-1 female mice were intermediate, significantly different from both WSP-1 and WSR-1, but more closely resembled the WSP-1 mice.

There was a highly significant effect of Line ( $F_{2,150} = 299.2, P < 0.001$ ). The WSP lines had markedly more pronounced withdrawal than the WSR lines for each of the four sex and replicate comparisons (Fig.

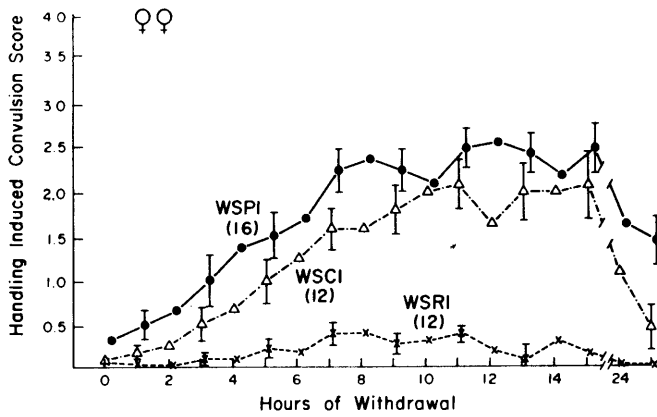


Fig. 2. Ethanol withdrawal convulsions (mean  $\pm$  SE) in female mice from  $S_{11}$  of the first replicate set of lines. WSP, withdrawal seizure-prone line; WSC, nonselected control line; WSR, withdrawal seizure-resistant line. Numbers of mice tested are shown in parentheses. Seizure scale is given in Table I. Mice were not scored during h 16–23, inclusive. All mice were made physically dependent by exposure to the same ethanol treatment.

3). While WSC-1 mice tended to resemble the WSP-1 line, the WSC-2 line tended to have less marked convulsions and resemble the WSR-2 line. This yielded a highly significant Line  $\times$  Replication interaction ( $F_{2,150} = 53.7$ ,  $P < 0.001$ ). The main effect of Replication and all other interactions were not significant (all  $F$ 's  $\leq 1$ ).

**Role of Body Weight.** Initial body weights and their changes over the course of treatment were also measured for the mice in each gener-

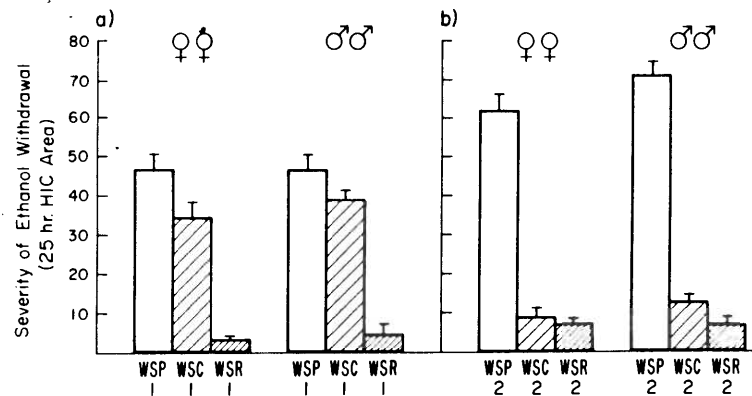


Fig. 3. Mean  $\pm$  SE area under the 25-h handling-induced convulsion curve (e.g., see Fig. 2) for all mice directly compared in  $S_{11}$ . (a) Mice from the first set of replicate lines; (b) mice from the second set of replicate lines. Each bar represents 12–17 mice drawn from as many families as possible within the line. All female mice were exposed to ethanol vapor in one chamber, and all male mice were exposed in another chamber.

ation (data not shown). The induction of physical dependence on ethanol produced only a small body-weight loss, in a range frequently intentionally employed to motivate mice for food reward experiments. Direct comparisons were made of body weight on day 1, when mice were not yet treated and were of very nearly identical ages. It was clear from these data that no important differences in body weight either before or after ethanol treatment have developed in the prone and resistant lines as a function of divergence for ethanol withdrawal severity.

**Estimates of Inbreeding.** Unavoidable deviations from the ideal within-family scheme described have increased the variance in family size from an ideal of zero about an ideal mean of two mice per family contributing to the next generation. Table V shows the total number of mice tested each generation and the actual number of families that contributed tested offspring to the next generation in each line. The latter number varies from 5 to 10 across generations. The actual number of mice ( $N$ ) contributing offspring is also shown. The mean and variance in reproductively participating family size are given, and the effective breeding population size ( $N_e$ ) was calculated as  $4N/(2 + \sigma_k^2)$  according to Falconer (1960). Assuming random mating between families within a line, the increase in inbreeding was cumulated by generation by line as  $\Delta F = 1/2N_e$ . It can readily be seen that inbreeding has increased at a rate of approximately 1.5% per generation. The WSP-2 line has a slightly higher rate of inbreeding than the other lines, which are all quite similar.

These values should be taken as minimum estimates, for in those instances where reserve matings were used, they were treated as independent families. Over generations, the average number of reserve family matings was 10–16% of the total in all lines, except in WSP-2, where it was 21%. The control lines, P1 and R1 lines have a slightly higher reproductive fitness (approximately 1 pup per litter) than the P2 and R2 lines (data not shown). Death of tested mice is a minor factor, since we typically lose only 1–3 mice of the 120–180 tested each generation. An additional consideration is the fact that the mating scheme described constitutes systematic cousin matings. Using the method suggested by Falconer (1960), Dr. Ronald Weigel calculated from paths the increased inbreeding that would be expected after 11 generations of cousin matings. The estimated increase in inbreeding would be 21%, which does not differ markedly from the estimates presented in Table V.

## DISCUSSION

These data demonstrate that withdrawal from ethanol as measured by the severity of handling-induced convulsions is clearly under genetic control, since a substantial heritability is revealed by the approximate 10-

fold response differential between WSP and WSR mice after 11 generations of selective breeding. This agrees with results we and others have reported from surveys comparing inbred strains of mice (Crabbe *et al.*, 1983c; Goldstein and Kakihana, 1974). In addition to diverging for withdrawal-convulsion severity measured as a joint function of intensity and duration (i.e., with an area measurement), the WSP and WSR lines also exhibit a difference in peak seizure intensity which is approximately five-fold in magnitude (data not shown).

In the WSP-1 and WSR-1 lines, blood ethanol concentrations at the time of withdrawal from chronic inhalation of ethanol may be diverging, at least in males. We have found the phenotypic correlation between the BEC at the time of withdrawal and the area under the 25-h withdrawal curve to be approximately 0.45–0.60 in unpublished studies with HS/lbg mice. When 20 inbred strains were examined, the genetic correlation between withdrawal severity and BEC at withdrawal estimated from strain means was  $r = 0.57$ , although a scatter plot suggested that this association was relatively weak (Crabbe *et al.*, 1983c). However, there is no evidence for divergence for BEC in the WSP-2 and WSR-2 lines. In many studies with Generations  $S_1$ – $S_{11}$ , we have found that WSP and WSR lines do not differ in the BEC attained after acute injections of ethanol or after a chronic series of injections (Kosobud *et al.*, 1984). On the whole, the evidence suggests that these lines do not differ principally in their blood or brain ethanol concentrations after acute or chronic ethanol administration.

Other studies have demonstrated that the WSP mice also have more marked tremor and degree of activity reduction in the hole-in-the-wall apparatus than the WSR mice after withdrawal from identical chronic ethanol administration (Kosobud *et al.*, 1984). Similarly, they show more pronounced withdrawal seizures after physical dependence on ethanol induced by liquid diet consumption, even though the WSR lines consumed more ethanol (Harris *et al.*, 1984). We take this to indicate that the phenomenon of ethanol withdrawal is multifactorial and that selection for one of the principal withdrawal responses in mice has resulted in a correlated response to selection in other alcohol withdrawal symptoms. This is hardly surprising, especially given the recent demonstration that a selective breeding program resembling that reported here has shown a response to selection in mice for a multifactorial index of ethanol withdrawal severity which is derived from several withdrawal symptoms (Wilson *et al.*, 1984). Indeed, the index of selection employed included handling-induced seizures and activity reduction in the hole-in-the-wall apparatus (Allen *et al.*, 1983). Systematic experiments will be necessary to characterize fully the differences between these selected lines and the WSP/WSR lines.

Naive mice show handling-induced convulsions, and these are exacerbated slightly by chronic pyrazole treatment (Crabbe *et al.*, 1980, 1981). Both these responses have been found to exhibit genetic variability, although the range of variability in a survey of 20 inbred mouse strains was small (Crabbe *et al.*, 1983c). Thus, the possibility exists that the present lines differ markedly in handling seizures in the absence of treatment or after chronic treatment with only pyrazole. This hypothesis was tested directly with mice from  $S_5$  and found not to explain differences of the magnitude reported here. While prone mice showed slightly more pronounced seizures than resistant mice after saline or pyrazole treatment, the differences are small relative to the differences induced by ethanol withdrawal (Crabbe *et al.*, 1983a; Kosobud *et al.*, 1984). The prone and resistant mice also differ only slightly (or not at all, depending upon the agent tested) in sensitivity to convulsions elicited by a number of convulsant agents and treatments (McSwigan *et al.*, 1984). In sum, these results argue that WSP and WSR lines have not diverged markedly as a result of selection simply for nervous-system sensitivity to experimental seizures. We feel that we have achieved our first goal with this model, namely, developing a general animal model of genetic susceptibility to ethanol physical dependence.

The second goal of the model we propose here may now be approached. Since not all close relatives of alcoholics develop the disorder, it would be useful to develop a predictive test capable of identifying the presence of heightened genetic susceptibility in individuals. If this were possible, preventive therapeutic measures could then be concentrated on an especially high-risk target population. While some studies with human populations offer promise for achieving this goal, progress toward understanding the physiological basis of such a predisposition will necessarily be slow in human research. It will be necessary to explore a number of relevant variables at the behavioral, neurochemical, and endocrine levels to try to identify which correlates of the selection index may be importantly related to withdrawal from ethanol physical dependence. We feel that the successful avoidance of high levels of inbreeding, the existence of replicated lines, and the relatively good reproductive fitness of the WSP and WSR lines after 11 generations of selection make them a useful model for pursuing such questions fundamental to furthering the understanding of the effects of chronic alcohol abuse.

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