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Evidence for Genetic Correlation of Hypnotic Effects and Cerebellar Purkinje Neuron Depression in Response to Ethanol in Mice

KAREN SPUHLER, BARRY HOFFER,[1] NORMAN WEINER AND MICHAEL PALMER†

Alcohol Research Center, Department of Pharmacology
University of Colorado School of Medicine, Denver, CO 80262
and †Department of Histology, Karolinska Institute, Stockholm, Sweden

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SPUHLER, K., B. HOFFER, N. WEINER AND M. PALMER. *Evidence for genetic correlation of hypnotic effects and cerebellar Purkinje neuron depression in response to ethanol in mice.* PHARMAC. BIOCHEM. BEHAV. 17(3) 569–578, 1982.—In the present study, we compared phenotypic differences in behavioral and neurophysiological responses to acute ethanol administration among eight inbred strains of mice. Genetic variation for behavioral sedation, as measured by loss of the righting reflex (sleep time) after a hypnotic dose of ethanol, was shown to be present among the inbred strain population. In addition, a large genetic component of variation in the depressant action of ethanol on the spontaneous discharge of cerebellar Purkinje neurons was found. Results from an analysis of covariance of the behavioral and electrophysiological phenotypes, measured on each mouse among the inbred strains, provided strong evidence for a high genetic correlation between sleep time and inhibition of cerebellar Purkinje neuron discharge in response to acute ethanol administration. Taken together with our previously reported data on ethanol-induced electrophysiological changes in selectively bred lines, the results described here strongly support the hypothesis that the cerebellar Purkinje neuron is one important locus for the acute soporific effects of alcohol.

Righting reflex Purkinje neuron Inbred strains Analysis of covariance Ethanol effects

GENETIC variation in behavioral sensitivity to the sedative effects of ethanol has been demonstrated in both mouse and rat laboratory populations (see [4]). For example, the genetic basis for variation in the effects of acute ethanol administration has been shown by selectively breeding two lines for differential sensitivity to an acute ethanol dose (IP), as measured by sleep time [6,31]. Sleep time, following a sedative dose of ethanol, is defined as the time interval from loss to recovery of the righting reflex. The selective breeding program has resulted in long sleep (LS) and short sleep (SS) lines that exhibit a marked difference in sleep time in response to a hypnotic dose of ethanol (4.4 g/kg for SS; 3.8 g/kg for LS) at the 24th generation of selection (32nd generation of breeding). There has been no overlap between the two response distributions of the selected lines since the 16th generation of selection [28,31].

Given the very large difference in behavioral sensitivity to ethanol expressed by the LS and SS mice, as well as the experimental evidence that the cerebellum is involved in the acute central nervous system effects of ethanol [8, 16, 22, 34, 40, 43], Sorensen *et al.* [41] studied the electrophysiological response of cerebellar Purkinje neurons (PN) to an acute ethanol dose applied locally in the LS and SS mice. Their findings indicated that LS mice were significantly more sensitive than SS mice in the magnitude of the mean dose to inhibit PN spontaneous discharge by 50% at the 17th generation of selection (25th generation of breeding). The activity of the PN of LS mice was depressed by a 30-fold lower dose of ethanol, on the average, than was that of SS mice. This difference in sensitivity between the LS and SS mice was apparently brain region- and depressant agent-specific, as no significant differences were observed in: (1) sensitivity of pyramidal neurons of the hippocampus to ethanol, or (2) PN responses to halothane administration [42]. Moreover, the mean dose of ethanol to depress the firing rate of the cerebellar PN by 50% in a sample of the parental heterogeneous stock of mice (HS/Ibg), from which the LS and SS lines were derived, was found to be precisely intermediate between that

[1]Send reprint requests to Dr. Barry Hoffer, Department of Pharmacology, C236, University of Colorado Health Sciences Center, 4200 E. Ninth Ave., Denver, CO 80262.

of the LS and SS mean doses [42]. Thus, a strong association between behavioral sensitivity to parenterally-administered ethanol and electrophysiological sensitivity of cerebellar PN to local ethanol was exhibited.

Recently, our laboratory has extended these findings by employing *in oculo* transplants of fetal cerebellar grafts of LS and SS mice [36]. In a design of within-line and between-line transplants of cerebellum to the anterior chamber of the eye of a recipient mouse, comparisons of the graft types demonstrated that the electrophysiological response of the transplanted cerebellum resembled that of the donor line in its sensitivity to perfused ethanol. The differential sensitivity of the LS and SS cerebellar grafts in response to the dose of ethanol perfused in the eye chamber corresponded to that observed in the *in situ* experiments of LS and SS cerebellum. Moreover, no differences in sensitivity for a given donor line were seen in within-line compared to between-line grafts. These data suggested that genotypic differences in the PN between LS and SS lines determine the ethanol sensitivity in the cerebellum. The epigenetic influences on neuronal sensitivity exerted by physiological functions of the host animal appear to be of much less importance in determining PN sensitivity [36].

Whereas these findings were strongly consistent with the hypothesis that there is a genetic component to the intrinsic PN differences in sensitivity to ethanol observed between the selected lines, and that there exists a genetic correlation relating the behavioral and neuronal sensitivity to the sedative effects of ethanol, additional genetic analyses are required in order to provide an adequate test of these hypotheses.

In the present communication, our experimental paradigm to test these hypotheses and to obtain an estimate of the genetic and environmental contributions to the variation in a sleep time and PN response to ethanol incorporates a comparison of a set of inbred strains. Analyses using inbred strains to estimate a genetic correlation has been presented in detail by Kempthorne [23] and recently discussed by Hegmann and Possidente [19]. A summary of this approach is presented next, in order to provide the rationale for these experiments.

If the number of inbred strains tested in the experimental design is sufficiently large, and the strains are measured concurrently and, in addition, differences in the environmental milieu to which the strains are exposed are minimized, then the measured differences among the inbred strains provide an estimate of the additive genetic (average effects of allelic differences at many different loci) variance as a component of the total phenotypic variance. The total phenotypic variance (V_P) of a trait is that variance which is observed in a sample of individuals from a designated population. It can be partitioned linearly into a genetic component (V_G) and an environmental component (V_E), plus a component due to genotype-environmental covariance ($2COV_{GE}$), such that $V_P = V_G + V_E + 2COV_{GE}$. The last component would be present when different genotypes are exposed to different environmental conditions. Its contribution to the phenotypic variance is minimized in laboratory studies by carefully controlling environmental factors that may confound the estimation of the other genetic and environmental components of variance. The genetic component of variance can be further partitioned into: (1) an additive component, due to the average effects of allelic differences summed over many gene loci (V_A); and (2) a component due to nonlinear interactions (V_{NA}) either between alleles at the same gene locus (dominance), or between different loci (epistasis), i.e., $V_G = V_A + V_{NA}$.

In an analysis of variance design, the between inbred strain component of variance is a function only of V_A, since members of a given strain are genetically alike (homozygous at effectively more than 95% of gene loci after 20 generations of inbreeding). In contrast, the within strain component of variance estimates only V_E. Any differences among members of the same strain are assumed to be due to environmental effects. In fact, it can be shown that the expected between strain component of variance, obtained from an analysis of variance, estimates twice the additive genetic variance ($2V_A$) in a population [23] where the gene frequencies equal one-half [30]. Thus, from the analysis of variance of inbred strains, the amount of phenotypic variance which is additive genetic, can be estimated. A useful population statistic, the heritability index [14], is defined as the ratio of additive genetic variance to total phenotypic variance, where $V_P = V_A + V_E$. The heritability (h^2) of a trait gives information about the magnitude of the genetic variation existing in the population and thus, about the causes of individual differences. Conversely, the ratio of the environmental component of variance to the phenotypic variance (e^2) provides information about the relative importance of environmental influences; thus,

$$h^2 = V_A/(V_A) + V_E), \qquad e^2 = V_E/(V_A + V_E)$$

where $h^2 + e^2 = 1$.

The range of h^2 or e^2 is between 0 and 1.

In an analogous fashion, the between strain component of covariance in an analysis of covariance is a function of the additive genetic covariance of the two traits, X and Y, considered jointly and thus, facilitates an estimate of the genetic correlation (r_A) of the two traits, where

$$r_A = \frac{\text{additive genetic covariance of X and Y}}{\sqrt{\text{(genetic variance X) (genetic variance Y)}}}$$

If an analysis of variance is computed on each trait separately (i.e., for X and for Y), and then computed on the sum of X and Y where phenotypic scores for X and Y are summed for each individual, an estimate of the additive genetic covariance can be obtained by using the additive genetic component of variance estimated from each separate analysis of variance of the traits as follows:

2 covariance (XY) = Variance (X + Y) − (Variance X + Variance Y).

The estimation of these genetic parameters (heritability index, environmental index and the genetic correlation) assumes that the inheritance of the traits is through multiple gene loci, which is defined as polygenic inheritance. In addition, constancy in environmental effects on different genotypes among the inbred strains is assumed. The estimates derived from the inbred strain analysis should be considered as upper-limit values for the parameters which describe genetic variation among the mice for ethanol sensitivity, since the inbred strains were chosen with *a priori* knowledge of the large differential phenotypic response of some of the strains (i.e., C57BL, C3H/2, BALB/c, and DBA/2) for sleep time.

METHOD

In order to sample the range of the hypnotic responses to acute ethanol administration, we chose eight different inbred strains which were closely related to the original inbred strains that were intercrossed to derive the heterogenous

stock (HS/Ibg; [32]). The LS and SS selected lines were initiated from the segregating population of HS/Ibg mice; therefore, our sampling of the eight inbred strains should theoretically yield a representation of the gene pool that was available for selection during the LS-SS breeding program.

The eight strains employed included C57BL/Crgl, ISBI/Crgl (from Cancer Research Laboratory, Berkeley, CA), C3H/2Ibg (from IBG, Boulder, CO), A/J, AKR/J, RIIIS/J, DBA/2J and BALB/cJ (from Jackson Laboratory, Bar Harbor, ME). The C57BL, ISBI and C3H/2 substrains used in this study were direct descendants of those originally crossbred to derive HS, whereas the remaining strains from the Jackson Lab were closely related substrains to those originally crossbred in establishing the HS mice. Male mice only were tested for their sleep time (in minutes), following a dose of 3.3 g ethanol/kg body weight, IP, as a 30% (w/v) solution in 0.9% saline. This dose was found to be effective in sedating the mice of all eight strains for at least 18 min, and was well below the LD_{50} value for all strains. All mice were tested for sleep time at 60–65 days of age, which is the age at which the LS and SS mice are routinely tested prior to choosing breeding pairs during each generation of selection.

Phenotypic scores on each mouse for sleep time and for the dose of ethanol needed to inhibit PN firing by 30–70% were obtained. Five mice within each strain were tested. The animals were taken from different litters in order to minimize litter effects contributing to the variance between strains. Mice were received at 5–6 weeks of age, and allowed to acclimate to our housing conditions for at least fourteen days prior to testing sleep time. All animals were housed under identical environmental conditions in large group cages of no more than six mice per cage, from which a random sample of five mice was chosen for experimentation. Purina Lab Chow and water were provided ad lib, and cages were changed with fresh bedding every four days. A twelve-hour light-dark cycle was employed.

Sleep Time Measurement

The protocol for estimating sleep time was similar to that used in the selective breeding of the LS and SS mice [31]. Sleeptime was measured as the time (minutes) between loss and recovery of the righting reflex after administration of ethanol. Mice were placed immediately in Plexiglas troughs after loss of the righting reflex. A mouse had to demonstrate the ability to right itself in the trough four consecutive times within a one-minute period, after the initial righting, in order for the first recovery to be counted as the end of the sleep time interval. All sleep time measurements were conducted between 8 a.m. and noon on different days. A maximum of eight mice from different strains were tested on any given testing day. Testing of strains for sleep time was randomized with respect to time of morning that sleep time was initiated on different days, as well as with respect to the day of testing.

After the sleep time measurements, mice were allowed to recover in their cages at a room temperature of about 27°C, to minimize the hypothermic reaction to ethanol [17, 26, 33, 38]. The recovery period for each mouse prior to measurement of PN sensitivity was at least four days, so that any development of acute tolerance [13] would have dissipated. Again, strain sampling for scoring mice on PN sensitivity was randomized with regard to time of day of recording. Diurnal variation in PN spontaneous discharge and the depression of firing rate by ethanol (see below) was not apparent in the inbred strain populations, while such variation for sleep time could be present [21]; thus, the time limitation was invoked for time of day of the behavioral testing.

Electrophysiological Recordings

The experimental procedures and protocols were similar to those we used previously for examining LS and SS mice [41]. Mice, weighing between 20 and 30 g, were anesthetized with 1.25 g/kg urethane injected intraperitoneally. The animals were intubated, placed in a stereotaxic instrument, and passively ventilated with oxygen. Body temperature was monitored and maintained at 37°C during recording. After the skull and dura mater over the cerebellar vermis were removed, the exposed surface of the cerebellum was covered with warm 2.5% agar in saline and the cisterna was opened at the foramen magnum to reduce brain pulsations.

The spontaneous action potential discharges of PN in lobules VI and VII of the cerebellar vermis were recorded extracellularly using two-barreled glass micropipettes, which were constructed as previously described [35,37]. The recording barrel was filled with 5 M NaCl. The drug barrel was filled with 750 mM ethanol in normal saline. PN were identified in each mouse by their anatomical location and characteristic discharge pattern of single and complex spikes [7]. Single action potentials of PN were monitored and photographed from an oscilloscope, separated from background activity and converted to constant voltage pulses with a window discriminator. The output of the window discriminator was connected to a digital computer (PDP-12, Digital Equipment Corp.; STA-1, Medical Systems Corp.) from which interspike interval histograms were constructed. The rate of spontaneous discharge of the PN was integrated over 1-sec intervals and displayed as spikes per sec on a strip chart recorder.

Ethanol was administered locally by pressure ejection [37] from the drug barrel of the micropipette. Pressure ejection was regulated by a pneumatic valve (1–35 pounds per square inch), and timing of the drug pulse was controlled by a crystal clock circuit (Medical Systems Corp., PPM-2). Previous studies have shown that drug administration with this technique is reproducible, and is linearly related to pressure and time of ejection [27, 37, 39]. Thus, the dose of ethanol can be expressed as the product of the pressure (pounds per square inch; psi) times the duration of ejection (seconds). There is little leakage of drug between ejection trials [37], and the released volume of drug in a 50 psi-sec dose is approximately 10^{-5} μl. The dose of ethanol eliciting a 30–70% depression in PN firing rate was considered valid for a given neuron only if the response could be replicated with a recovery to control firing rate in at least two consecutive trials.

Ratemeter records were analyzed for changes in neuronal discharge rate induced by ethanol application, as previously described [35]. The data from the ratemeter records were digitized with a Tektronix graphics tablet and fed to a Data General NOVA 3/12 computer. The resulting output was quantified as the percentage of depression elicited by local ethanol application. Previous reports from our laboratory have validated this approach to quantitative microadministration of drugs [15,35]. Using these techniques drug responses can be evaluated independently of variations in background discharge [15]. Previously described controls were used to test for ejection pressure artifacts and control for osmolarity of the ethanol solution [37,41]. The average

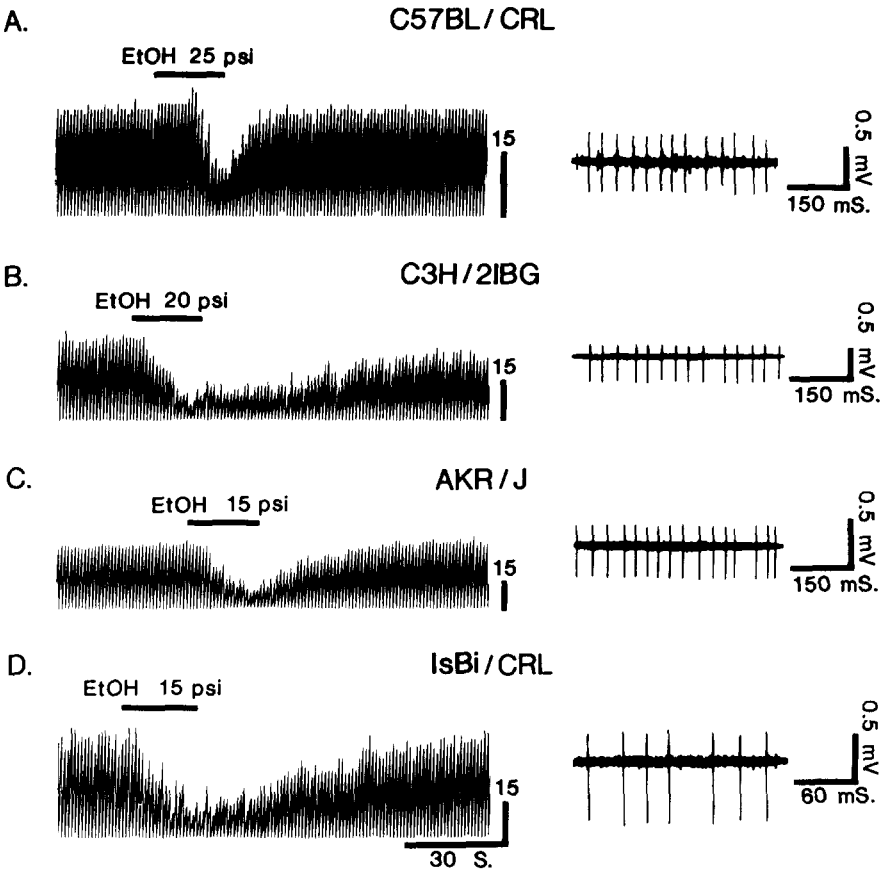

FIG. 1. Sample ratemeter records (left) and spike records (right), and examples of the effects of micropressure ejected ethanol (shown in the ratemeter record) for the C57BL/Crgl (A), C3H/2Ibg (B), AKR/J (C), and ISBI/Crgl (D) inbred strains of mice. In this figure and in Fig. 2, the number after the ETOH (ethanol) indicates ejection pressure in pounds per square inch (psi); the duration of ejection is indicated by the underlying bar, and the ratemeter ordinate is expressed in spikes per second. In all four cases, the neurons generate a rapid regular spontaneous discharge, which was slowed by the local pressure ejection (15–25 psi) of ethanol (left). These cells produced extracellularly recorded action potentials which had normal waveforms and high signal-to-noise ratios (right).

discharge rate before, during and after ethanol administration was computed by integrating the area under the designated ratemeter curve and the percent change in spontaneous discharge was estimated. To insure reliability of PN sensitivity measurements, all neurons had to exhibit a stable discharge pattern during the control period, as determined by interspike interval histograms after ethanol administration, and subsequent recovery. After a determination of the dose-response relationship for each PN sampled, the dose that most closely approximated a 50% depression but always fell within a 30–70% depression of firing rate, was selected as a measure of the neuron's responsiveness. The 30–70% window was selected in order to avoid artifacts due to ceiling or threshold effects. Averaging the sample of neurons for each mouse yielded a mean dose in psi-sec which inhibited the firing rate of the neuronal population by approximately 50%.

For each of the five mice tested for sleeptime within a given inbred strain, five Purkinje neurons were tested for the psi-sec dose of ethanol to inhibit their firing rate by 30–70%. In order to minimize the variability in release of ethanol between separate micropipettes which could contribute to the variance between inbred strains, a given micropipette on a single day of recording was used to test at least two PN in each of several inbred strains (e.g., a micropipette would be used to study ethanol effects on two neurons in one C57BL mouse, and used later to study two neurons in one DBA/2 mouse).

RESULTS

Neuronal Sensitivity to Ethanol

A total of 200 cerebellar Purkinje neurons, recorded from 40 mice (8 inbred strains, 5 mice per strain, 5 neurons per mouse) were used for analysis. The extracellularly recorded action potentials from these neurons displayed typical waveforms and high signal to noise ratios (Fig. 1 and 2, insets). The local application of ethanol produced depressions of PN firing in all eight inbred strains (Fig. 1 and 2). These effects were qualitatively similar to those reported previously in LS, SS and HS mice [42], and in Sprague-Dawley rats [40].

The within-strain variances in the dose of ethanol to in-

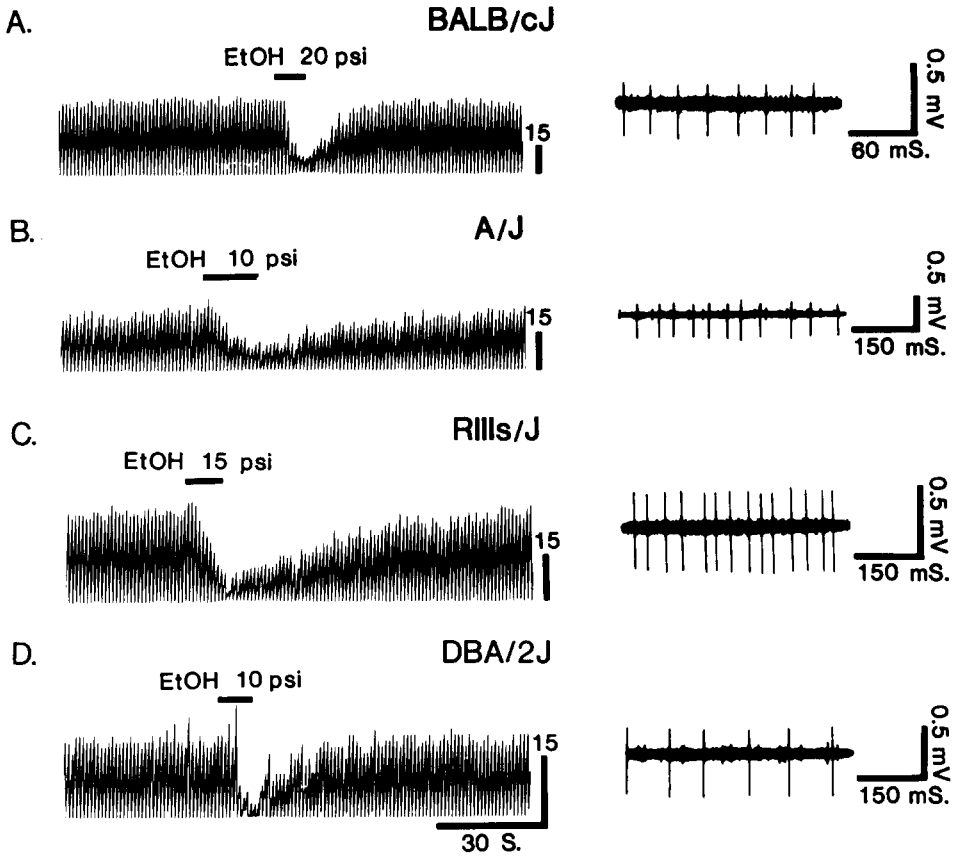

FIG. 2. Sample ratemeter records (left), spike records (right) and examples of the effects of micropressure ejected ethanol (shown in the ratemeter record) for BALB/cJ (A), A/J (B), RIIIS/J (C), and DBA/2J (D) inbred strains of mice. In all four cases, the neurons generated a regular and rapid discharge which was slowed by the local pressure ejection (10–20 psi) of ethanol (left). These cells produced extracellularly recorded action potentials which had normal waveforms and high signal-to-noise ratios (right).

hibit PN firing were homogeneous, as determined by Cochran's test for heterogeneity of variance ($C_{obs} = 0.327 < C_{0.05} = 0.391$, with 4 *df*). A nested analysis of variance (ANOVA) of the ethanol dose in psi-sec inhibiting PN in the eight inbred strains indicated a significant difference in variance between the strains (S^2_A) in PN sensitivity, $F(7,32)=34.9$, $p<0.001$, as illustrated in Table 1. The variance between mice within strains (S^2_B) did not account for a significant proportion of the total variance in neuronal sensitivity, $F(32,160)=0.7$, NS. By pooling the variance components between mice-within strains ($S^2_B=-0.616$), and between neurons-within mice ($S^2_e=10.84$), an estimate of the reliability of the electrophysiological recordings, based upon the ratio ($\rho_K=S^2_A/S^2_A+S^2_e/K$, where K=number of neurons per strain, 25) of the between-strain variance component ($S^2_A=10.40$) to the remaining pooled within-strain variance component ($S^2_e=10.32$), was derived, using the mean square values (MS) of the ANOVA, Table 1 [44]. The reliability coefficient was 0.96, demonstrating a high level of internal consistency in the measurement. Thus, experimental variance due to preparative surgery of the mice for *in situ* recording, as well as the use of a series of different micropipettes for recording from different mice, was appropriately controlled. From this result, it was deemed appropriate to use the mean dose of ethanol in psi-sec to inhibit firing of the five sampled PN per

TABLE 1

HIERARCHICAL ANALYSIS OF VARIANCE*† OF THE ETHANOL DOSE IN PSI-SEC UNITS INHIBITING PURKINJE NEURON FIRING RATE BY 30–70% IN EIGHT INBRED STRAINS OF MICE

Source of Variation	*df*	SS	MS	F
Between Strains	7	1892.76	270.40	34.9‡
Between Mice, Within Strains	32	248.27	7.76	0.7
Between Neurons, Within Mice, Within Strains	160	1743.04	10.84	
Total	199	3875.08		

*The design included five mice measured within each of eight inbred strains and five independent Purkinje neurons measured within each mouse to yield 200 total neurons recorded.

†The reliability coefficient, based upon the ratio of the between-strain component of variance to the pooled between-mice and between-neuron, within-mice components of variance, was estimated as $\rho_K=0.96$. This provides a measure of the internal consistency of the electrophysiological recording of neuronal inhibition by ethanol, employing pressure ejection from the micropipettes.

‡$p<0.001$.

TABLE 2

MEAN RESPONSE OF EIGHT INBRED STRAINS TO AN ACUTE DOSE OF ETHANOL: BEHAVIORAL SENSITIVITY (SLEEP TIME) AND CEREBELLAR NEURONAL SENSITIVITY

Phenotype	Inbred Strain*							
	C57BL	C3H/2	AKR	ISBI	BALB/c	A	RIIIS	DBA/2
Sleep time†	28.0 ± 4.7	36.8 ± 5.8	54.2 ± 7.1	64.4 ± 4.8	71.8 ± 4.5	74.0 ± 6.7	76.8 ± 4.2	83.4 ± 7.0
Dose to Inhibit‡ Purkinje Neuron Firing Rate	524 ± 38	423 ± 24	325 ± 16	232 ± 6	175.1 ± 9	153 ± 13	150 ± 4	98 ± 7

*C57BL and ISBI were from CRL, Berkeley; C3H/2 was from IBG, Boulder; AKR, A, BALB/c, RIIIS, and DBA/2 were from Jackson Lab, Bar Harbor.

†Each value represents the mean sleep time in min ±SEM of 5 mice per strain. The dose to elicit sedation was 3.3 g/kg mouse, IP.

‡Each value represents the mean dose ±SEM in PSI-SEC units of effective doses from 5 mice per strain. Effective doses were calculated as those which caused a 30–70% inhibition of neuronal firing rate averaged over five Purkinje neurons per animal. Ethanol was locally applied to the neuron from one barrel of a micropipette using pressure ejection, as the firing rate was recorded simultaneously through the recording barrel of the microelectrode.

mouse as the best estimate of the animal's electrophysiological response to locally applied ethanol.

Behavioral Sensitivity to Ethanol

Table 2 shows the mean behavioral responses of the eight inbred strains to an acute dose of ethanol (3.3 g/kg body weight). The variances of the sleep time measurements of the inbred strains were homogeneous, as determined by Cochran's test ($C_{obs} = 0.193 < C_{0.05} = 0.391$, 4 *df*). The C57BL strain exhibited the shortest mean sleep time, whereas the DBA/2 strain slept the longest, when compared to the other strains. The sleep times of BALB/c, A, and RIIIS strains were not significantly different from one another, and approached the DBA/2 sleep time. The C3H/2 strain tended to have only a slightly longer sleep time than C57BL, and that of AKR was intermediate. The ISBI strain showed a higher sensitivity than AKR, but was somewhat less sensitive than either BALB/c, A or RIIIS. Overall with regard to mean sleep times in response to ethanol, the eight inbred strains formed three general clusters: C57BL-C3H/2, AKR-ISBI, and BALB/c-A-RIIIS-DBA/2, rank-ordered from lesser to greater sensitivity. For C57BL, C3H/2, BALB/c and DBA/2, the relative behavioral sensitivity to ethanol among strains is similar to that of earlier reports [3, 5, 20, 28]. A replication of the sleep time in the eight inbred strains, using six mice per strain in a subsequent study, has yielded a repeatability measure (correlation of the two independently sampled means for each strain) of 0.93. This high value for the coefficient indicates reasonable reliability of the behavioral measurements among the strains.

Phenotypic Correlation of Behavioral and Neuronal Sensitivity to Ethanol

The averaged mean dose to inhibit PN firing rate of each of the eight inbred strains is also shown in Table 2. A similar ranking in this neuronal phenotype among the strains is observed when compared to sleep time. A five-fold range of PN sensitivity to ethanol among the strains was observed. The mean dose of ethanol to inhibit the PN of C57BL by 50% was significantly larger than the doses for the other strains. This finding indicated that C57BL possessed PN that were much less sensitive to acute ethanol than the other strains. DBA/2 was found to be the most sensitive in PN response to ethanol. Again, the inbred strains tended to form clusters in their relative PN sensitivity to ethanol, as follows: C57BL-C3H/2, AKR-ISBI, BALB/c-A-RIIIS and DBA/2. The sensitivities of BALB/c, A, and RIIIS are very close in magnitude and less than DBA/2.

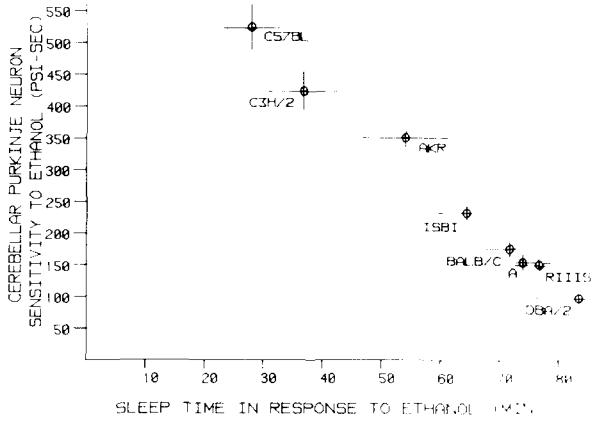

FIG. 3. The correlation of sleep time and Purkinje neuron sensitivity to acute ethanol among eight inbred strains of mice, five mice per strain. The ordinate represents the pressure ejection dose (in pounds per square inch-seconds) applied from a two-barreled micropipette, that caused approximately 50% inhibition of Purkinje neuron firing rates (see Methods). Each point in the plot represents the mean dose of twenty-five Purkinje neurons, pooled over five mice within a strain. The abscissa represents the length of time (in minutes) that the animals lost their righting reflex (sleep time) after receiving 3.3 g/kg ethanol IP. Each point represents the average of five sleep time scores, one for each animal studied.

In Fig. 3, the mean responses to ethanol in terms of behavioral sensitivity and of neuronal sensitivity are depicted in a bivariate plot. The sleep times reported here are the mean responses of five animals per strain. The neuronal sensitivity for each strain is the mean response of 25 neurons; these neurons were recorded from the cerebella of the same

TABLE 3

ANALYSIS OF COVARIANCE OF BEHAVIORAL SENSITIVITY (SLEEP TIME) AND BRAIN (CEREBELLAR PURKINJE NEURON INHIBITION) SENSITIVITY IN RESPONSE TO AN ACUTE DOSE OF ETHANOL IN EIGHT INBRED STRAINS OF MICE

Phenotype	Source of Variation	*df*	SS	MS	F
Sleep time†	Between Strains	7	13846.17	1978.02	12.22*
	Within Strains	32	5179.60	161.86	
	Total	39	19025.77		
Ethanol Dose to Inhibit Neuronal Firing Rate‡	Between Strains	7	378.55	54.08	38.45*
	Within Strains	32	49.66	1.55	
	Total	39	428.22		
Synthetic Variable from Summing Scores of the Two Phenotypes	Between Strains	7	18288.57	2612.65	16.66*
	Within Strains	32	5017.65	156.80	
	Total	39	23306.22		

*$p<0.001$.

†The sleeptime scores for each mouse was measured in minutes from loss to recovery of righting reflex elicited by a 3.3 g/kg mouse dose, IP.

‡The ethanol dose in psi-sec units to inhibit Purkinje neuron firing rate by 30–70% was converted to the measure, (1000 (1/psi-sec)), in order to derive a positive association between the variables, measuring behavioral and neuronal sensitivity for estimation of components of genetic variation from the ANCOVA.

animals used for the sleep time measurements. There is a very strong linear relationship between the mean sleep time in response to ethanol and the mean cerebellar PN sensitivity to ethanol among the eight inbred strains. A negative relationship is observed as a higher dose of ethanol is required to depress the firing rate of the neurons of a strain which sleeps for a shorter period of time following a hypnotic dose of ethanol. The correlation coefficient of this function is −0.997, a value significantly different from 0, but not from 1. It is noteworthy that the points for the LS and SS mice, as taken from Sorensen *et al.* [41], would fall outside the bivariate distribution range of the inbred strains depicted in Fig. 3. The LS, with an average sleep time of 2.4 hrs at a 4.1 g/kg IP ethanol dose, exhibited a mean PN sensitivity dose of 29±5.5, and the SS, with an average sleep time of 11 min, exhibited a mean PN sensitivity dose of 888±147. This relationship is consistent with the fact that the selected lines have been bred approximately 20 generations for a specific combination of gene complexes that express the extreme phenotypic values, whereas the inbred strains have had no selection pressure applied during their breeding for these measures. Clearly, genetic differences exist between the inbred strains for ethanol sensitivity. However, the fixation of gene complexes within the strains for ethanol sensitivity occurring during their historical development was apparently a chance event, consistent with random genetic drift. The range of sleep time scores among the eight inbred strains does approximate that observed in sampling of HS/Ibg, the foundation stock for LS and SS lines [28,31]. When the values for all strains were pooled, the mean (61 min) of the 40 mice in this study was very close to the sleep time observed in male HS mice in response to a 3.3 g/kg dose of ethanol prior to the initiation of the selection program.

Analysis of Covariance of the Behavioral and Neuronal Sensitivity to Ethanol

Table 3 shows the analysis of covariance for sleep time, the ethanol dose to inhibit PN firing, and the sum of the two phenotypes. The between-strain component of variance, estimated from the difference in the ANOVA mean squares between and within strains, weighted by the reciprocal of the number of mice per strain, comprises a significant portion of the total variance, as indicated by a highly significant F value in each case ($p<0.001$ for sleep time, PN sensitivity, and the phenotypic sum). From this analysis the significant between-strain component of variance indicates a genetic contribution to the phenotypic variation for behavioral and PN sensitivity to ethanol. The estimation of genetic and environmental parameters for sensitivity to ethanol among the inbred strains yielded the following values:

Phenotype	Heritability	Environmental Effect
Sleeptime	0.53 ± 0.14	0.47 ± 0.18
PN Firing Inhibition	0.77 ± 0.11	0.23 ± 0.18

A large amount of additive genetic variance for both sleep time and PN sensitivity in response to ethanol is evident in this population of inbred strains.

From the analysis of covariance, the estimate of the genetic correlation of sleep time and PN sensitivity was 0.95± 0.12. This finding suggests that many of the same genes influence both behavioral and neuronal sensitivity to ethanol. The environment correlation was estimated as −0.21, a value markedly lower than the genetic correlation.

The parameters which have been estimated can be used to derive an expected phenotypic correlation ($\hat{r}_p$) that might be observed in a segregating population of mice, where random mating was closely approximated. The expected value is defined as follows:

$$\hat{r}_p = h_x h_y r_A + e_x e_y r_E$$

where h_x and h_y are $\sqrt{\text{heritability index}}$ of traits x and y, respectively,

e_x and e_y are $\sqrt{\text{environmental index}}$ of traits x and y, respectively, and

r_A = additive genetic correlation
r_E = environmental correlation

In the inbred strain population, the expected phenotypic correlation from the parameter estimates is +0.54. Thus, in a random sample of offspring from litters that make up HS/Ibg in any one generation, we would expect a correlation of 0.54 between sleep time and PN sensitivity in response to ethanol. This moderate correlation, indicating a significant association between the two traits in a fairly large segregating population, is a function, then, of the amount of additive genetic variance present for each trait, the genetic correlation between the two traits, as well as the analogous environmental parameters.

DISCUSSION

In the present study, we compared phenotypic differences in behavioral and neurophysiological responses to acute ethanol administration among eight inbred strains. Genetic variation for behavioral sedation, as measured by loss of the righting reflex (sleep time) after a hypnotic dose of ethanol, was shown to be present in the inbred strain population. In addition, a strong genetic component of variation in the depressant action of ethanol on the spontaneous discharge of cerebellar Purkinje neurons was found. Results from an analysis of covariance of the behavioral and electrophysiological phenotypes, measured on each mouse among the inbred strains, provided strong evidence for a high genetic correlation between sleep time and inhibition of cerebellar Purkinje neuron discharge rate in response to acute ethanol administration.

The differences in behavioral sensitivity of the inbred strains reported here is consistent with previous reports for several of the strains (i.e., C3H/2, C57BL, BALB/c and DBA/2), in regard to relative ranking of sensitivity in response to the given sedative parenteral dose of ethanol [2, 3, 5, 20, 29]. Although the actual mean sleep time value of a particular strain will shift upwards or downwards in relation to the magnitude of the ethanol dose within a large range of the dose-response curve, the relative response of each strain should remain the same in comparison to the other strains. At the lower end of the curve (<3.0 g/kg), the differences in mean sleep time in response to ethanol become attenuated, and the sensitivity rank order of strains can change [2,5]; however this dose range is well below the test dose given in this study. The C57BL strain has exhibited the lowest sensitivity to an acute hypnotic dose of ethanol (≥3.0 g/kg) consistently in different laboratory testings, while BALB/c and DBA/2 have shown the greatest sensitivity. C3H falls intermediate to C57BL and DBA/2 (cf. present results with summary of studies in [4]). In the present study, a higher proportion of the eight inbred strains falls toward the direction of greater sensitivity to ethanol in their mean sleep time and PN sensitivity.

In addition to the strong relationship between behavioral and cerebellar PN sensitivity to ethanol administration, the phenotype measured as alcohol preference (see [4, 13, 18]) might also be related to brain sensitivity, since studies have indicated that the relative ranking of C57BL, C3H/2 and DBA/2 for alcohol preference parallels their ranking for sleep time in response to a parenteral dose of ethanol. Moreover, there are lines of rats which have been selectively bred for differences in ethanol preference. Both P and NP lines described by Li and coworkers [24,25], and the AA and ANA lines described by Eriksson [10, 11, 12] also show differential acute sensitivity to ethanol, although the difference is far smaller than that of preference. In each case, the alcohol preferring line is less sensitive than the ethanol nonpreferring line. The interrelationship of these phenotypes alcohol preference, loss of righting reflex after a hypnotic dose of ethanol, and electrophysiological differences in the depressant effects of ethanol should be studied further both in the inbred strains and selected lines, using genetic analysis.

The magnitude of the heritability index for sleep time is consistent with the results of successful breeding reported for high and low sensitivity to the hypnotic effects of ethanol in the LS and SS mice, derived from the segregating population of HS/Ibg. McClearn [28] reported an estimate of average realized heritability of 0.18 for the first five generations of selection in the LS and SS lines. The same value was estimated recently for the 24th generation of effective selection. However, the contribution to phenotypic variation from environmental effects certainly cannot be disregarded; factors in the environment, which as yet have not been discovered, may play a role in the sedative effects of acute ethanol as measured in the mice. It is not uncommon to find a significant environmental component contributing to variation in behavioral traits. This may be partially due to a less consistent measurement of the trait, relative to traits that are more physicochemical in nature, as well as to a greater plasticity in behavior and a much larger number of variables determining the range of response in the behaving animal.

The heritability estimate for PN sensitivity is quite high, and demonstrates that there is a large amount of additive genetic variance present in the physiological response of the cerebellar neurons to ethanol. The PN response to ethanol is less influenced by enviornmental factors than sleep time under our experimental conditions. However, it should be emphasized that estimation of these parameters of genetic and environmental variances are population-specific. The magnitude of the values are conditional on both the strain genotypes tested and the particular laboratory environment.

The genetic correlation of these two traits implicates a common genetic mechanism underlying the expression of the two parameters of sensitivity to ethanol, such that genes controlling the cerebellar PN response to acute ethanol also determine, to a large degree, the sleep time response to an acute dose of ethanol. A genetic correlation also could occur when separate gene loci, which have a major contribution to the expression of the two traits, are closely linked on the same chromosome. Recombinant inbred strains [1,9] are a very powerful genetic tool for estimating genetic correlation, and assessing further whether the genetic association of the two traits is related to pleiotropy (common genes governing more than one trait) or closely linked major genes. The recombinant inbred strains could be used to determine the linkage map distance between ethanol related and marker genes on the same chromosome, that is a function of the frequency of crossing-over of segments of genetic material between homologous chromosomes at meiosis.

The differential sensitivity of cerebellar Purkinje neurons in the various strains of mice cannot be generalized to neurons from all brain regions. We have shown that hippocampal CA1 pyramidal neurons have similar sensitivities to ethanol in LS, SS and HS mice [42]. This observation,

taken together with the findings of a genetic correlation between ethanol-induced loss of righting reflex and inhibition of PN firing, might suggest that changes intrinsic to PN function mediate the behavioral changes. However, at least two alternate hypotheses, which are not necessarily mutually exclusive, could also be put forth. The PN from the cerebellar vermis might regulate some small component of the righting reflex without actually controlling the behavioral outcome in total. Then, the ethanol-induced inhibition of PN activity would result in disruption of activities of a large number of neuronal circuits with which the cerebellar neurons interact. If some of these pathways mediate the righting reflex, then a disruption of their activities could result in the loss of this behavior with administration of ethanol. Alternatively, cerebellar PN might represent a subgroup of a class of neurons having a common developmental origin, all of which would express the differential sensitivity to ethanol observed in PN. Neurons of this group, other than PN, might mediate the righting reflex and also show a PN-like sensitivity to ethanol. However, it has been shown in ablation studies that the cerebellar output mediated via PN plays a major role in the righting reflex [7].

In conclusion, these findings on the effects of acute administration of ethanol demonstrate that a strong genetic association exists between sensitivity in the central nervous system and the sleep time response, and that the brain sensitivity is, at least in part, localized to cerebellar neuronal circuitry. A starting point for determining the physiological mediation of a complex behavior related to ethanol intoxication is provided in this study by genetic analysis of the covariation of phenotypic responses. Further research to assess if the site of genetic alterations in neuronal response to ethanol is intrinsic to the PN (postsynaptic) or related to input neurons to the cerebellum terminating on PN (presynaptic) should prove interesting. In addition, it should be determined if groups of neurons, located outside of the cerebellum, show a differential sensitivity to ethanol that would be similar to that observed in Purkinje neurons. Finally, an elucidation of differences in ethanol-membrane interactions between genetically different strains of mice may prove fruitful for determining the biochemical mechanism of action of alcohol.

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