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Ethanol disordering of spin-labeled mouse brain membranes: Correlation with genetically determined ethanol sensitivity of mice

(electron spin resonance/fluidity/genetics)

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ABSTRACT **Disordering of brain and erythrocyte membranes by ethanol *in vitro* was measured by ESR using 5-doxylstearic acid as spin label. Synaptosomal plasma membranes and erythrocyte membranes were isolated from two lines of mice developed, by selective breeding, for differential sensitivity to hypnotic effects of ethanol. Membranes taken from alcohol-sensitive "long-sleep" mice were more strongly disordered by ethanol *in vitro* than were membranes from alcohol-resistant "short-sleep" mice. Furthermore, within a population of genetically heterogeneous mice, the most ethanol-sensitive animals had the most ethanol-sensitive synaptosomal plasma membranes. *In vivo* sensitivity of the individual mice was evaluated by measuring brain ethanol levels at a precise behavioral end point, recovery from ataxia. The data extend our previous observations of correlations between *in vitro* and *in vivo* effects of ethanol and suggest that membrane disordering may be a primary mechanism of acute effects of ethanol.**

The physical action of ethanol on cell membranes can be demonstrated *in vitro* by a change in the order parameter, S, of spin-labeled biomembranes. The order parameter represents the degree of restraint imposed on molecular motion by the membrane structure in the environment of the probe. We have previously shown that addition of ethanol to spin-labeled mouse synaptosomal and erythrocyte plasma membranes *in vitro* disorders the membranes. This effect is concentration-related over the range 10–350 mM (1) and is stronger in the membrane core than near the surface (2). To address the question of whether the disordering is directly related to the behavioral effects of ethanol, we seek pharmacological, temporal, and genetic correlations between *in vivo* and *in vitro* responsiveness to ethanol. Pharmacological correlations are studied by comparing potencies of drugs *in vivo* and *in vitro*. We have shown that the membrane-disordering action of a series of aliphatic alcohols correlates well with their potency *in vivo* as measured by the ED_{50} for loss of righting reflex in mice (3). Temporal correlations are studied by comparing the time courses of altered drug sensitivity (e.g., during chronic administration) and of the *in vitro* effect. We have reported that membranes isolated from mice that had been treated chronically with ethanol were resistant to ethanol added *in vitro*, indicating that mice and membranes had developed tolerance in parallel (4).

Here we report a genetic correlation. We measured the *in vivo* and *in vitro* effects of ethanol in genetically different populations of animals. We used the "long-sleep" (LS) and "short-sleep" (SS) lines of mice (5) as well as the genetically heterogeneous population designated HS (6), which was the foundation stock for the selective breeding program. The LS and SS lines were selectively bred by McClearn and Kakihana (7) for differential sensitivity to ethanol, the duration of loss of righting reflex ("sleep time") being used as selection criterion. The sleep times of the two lines have progressively diverged over more than 18 generations (5). We show here that membranes prepared from mice of the LS and SS lines differ in sensitivity to ethanol.

To test the generality of this observation, we chose ethanol-sensitive and ethanol-resistant HS mice. The HS mice were developed by balanced crosses of eight inbred strains of mice. Samples from this population represent many possible combinations of a large, stable pool of genes. Individual HS mice differed considerably in the brain ethanol concentration at which they recovered from ataxia, and synaptosomal plasma membranes prepared from ethanol-sensitive mice were more strongly disordered by ethanol *in vitro* than were membranes from ethanol-resistant mice. This observation suggests that membrane disordering may mediate some aspects of alcohol intoxication.

METHODS

Mice. Male mice of the LS and SS lines (25th generation) and the HS stock were obtained from the Institute for Behavioral Genetics (Boulder, CO). They were tested at age 7–9 weeks.

Test of Ataxia. HS mice were randomly assigned to groups of 18 for testing on three different days. The mice were injected intraperitoneally with ethanol, 2.5 g/kg given as 25% (wt/vol) in saline. This dose produced obvious ataxia. When the mice appeared to be recovering, they were tested repeatedly by placing them on a horizontal dowel. The moment of recovery from ataxia was defined as the time when the mouse was able to remain on the dowel for 30 sec. The mouse was then decapitated and the brain was homogenized in 9 vol of 10% sucrose. An aliquot was taken for an immediate enzymatic assay of ethanol (8) in order to identify the three highest and three lowest brain ethanol concentrations among the 18 mice tested that day. Membranes were prepared from nine sensitive and nine resistant mice.

Preparation of Membranes. Synaptosomal plasma membrane fractions were prepared from whole brain homogenates by the flotation-sedimentation procedure of Jones and Matus (9), as described (1). Brains from two or three LS or SS mice were pooled, and three such pools were used for each line. In testing the HS mice, membrane fractions were prepared from individual mice; the three most sensitive and three most resistant mice each day were used. Synaptosomal plasma mem-

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Abbreviations: LS, long-sleep; SS, short-sleep; HS, heterogeneous stock.

brane preparations were frozen in liquid nitrogen until used; they were thawed before spin-labeling.

Erythrocyte ghosts were prepared from LS and SS mice, by a modification of the method of Kury and McConnell (10), from tail blood and trunk blood obtained after decapitation. Blood was pooled from the same mice used for the pooled brains. The ghost suspension was brought to 80% particulate matter by volume before spin labeling. Erythrocyte membranes were used within 24 hr of preparation.

ESR Measurements. The membranes were spin-labeled with 5-doxylstearic acid as described (2); we used 75 μg of spin label per 200 μl of membrane suspension. Replicate samples (15–25 μl) were stored at 4°C overnight with buffer or different concentrations of ethanol. One to three such samples at each concentration were made from each of the six pooled spin-labeled preparations from LS or SS mice. For HS mice, one spin-labeled preparation per brain was used to make one or two samples at each ethanol concentration. The final protein concentration was 20 mg/ml, determined by the method of Lowry *et al.* (11). The samples were placed in capillary tubes and inserted in the cavity of a Varian E-104A ESR spectrometer thermostated to 37°C (except in the experiment in which temperature was varied). The digitized spectral data were used to compute the polarity-corrected order parameter, *S*, according to Hubbell and McConnell (12), as described (2). The order parameter for each sample was the mean of three or more scans on the same sample.

RESULTS

Membrane Fluidity in LS and SS Mice. The order parameters of erythrocyte and synaptosomal plasma membranes isolated from the LS and SS lines of mice were similar in the absence of added ethanol. For erythrocyte membranes, the mean (± SEM) order parameter for three pooled blood preparations was 0.608 ± 0.005 for LS mice and 0.605 ± 0.005 for SS mice. The order parameters for the corresponding synaptosomal plasma membranes were 0.595 ± 0.001 for LS and 0.594 ± 0.001 for SS mice.

On addition of ethanol, a difference between the LS and SS lines was revealed. Ethanol disordered synaptosomal and erythrocyte membranes from both lines of mice, but membranes from LS mice were more affected than were those of SS mice. For erythrocyte membranes (not shown), the ethanol-induced change in order parameter showed a significant (genetic) line effect ($P < 0.05$) and ethanol effect ($P < 0.05$) in a two-way analysis of variance. For synaptosomal plasma membranes (Fig. 1), both effects were highly significant: for the line effect, $P < 0.001$; for the ethanol effect, $P < 0.001$.

To study the specificity of the membrane response to ethanol, we investigated the effect of another agent that can change membrane order—namely, temperature. Order parameters were determined with 5-doxylstearic acid at four temperatures between 30 and 39°C. Membranes from the LS and SS lines did not differ in response to temperature (Fig. 2).

Brain Ethanol at Threshold in HS Mice. HS mice recovered from ataxia at brain ethanol concentrations ranging from 39 to 62 μmol/g wet weight of brain, confirming that they had the desired broad distribution of sensitivity to ethanol. The nine mice selected as the ethanol-sensitive group had a mean (± SEM) brain ethanol concentration of 41.8 ± 0.58 μmol/g; the resistant group had 57.0 ± 1.02 μmol/g. The selected sensitive animals remained ataxic for 122 ± 5.0 min (mean ± SEM), but the resistant mice recovered at 72 ± 5.2 min.

ESR Results in HS Mice. Synaptosomal plasma membranes from each of the selected HS mice were clearly disordered by ethanol *in vitro*, in a concentration-related way. Membranes from individual mice of the sensitive group were more sensitive to ethanol *in vitro* than were membranes of mice from the ethanol-resistant group (Fig. 3). Analysis of variance for the combined data showed significant effects on the decrease in order parameter from base line according to group ($P < 0.001$) and ethanol concentration ($P < 0.001$). The base-line order parameters did not differ significantly between groups; they were 0.590 ± 0.0002 (mean ± SEM) for ethanol-sensitive and 0.590 ± 0.0002 for ethanol-resistant mice.

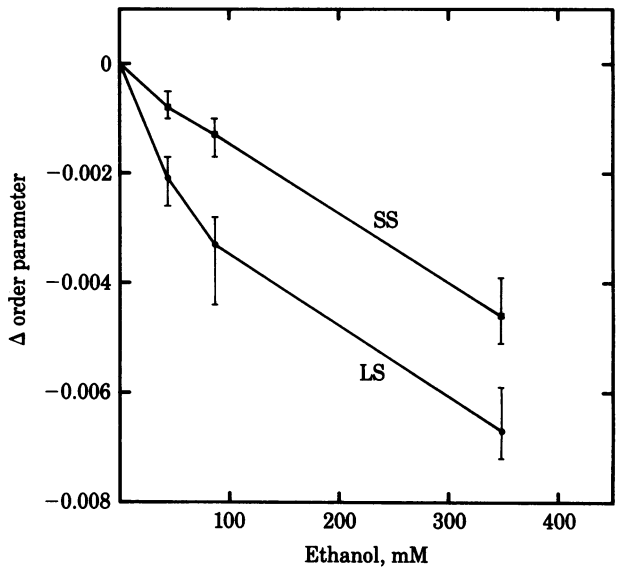

FIG. 1. Change in order parameter on addition of ethanol to isolated synaptosomal plasma membranes of LS and SS mice. Abscissa: concentration of ethanol in which the samples were incubated and tested. Δ = difference between the order parameter of each sample in ethanol and its base-line order parameter. Mean base-line order parameters are listed in the text. ●, Membranes from LS mice; ■, membranes from SS mice. Points are means of three separately spin-labeled preparations; vertical bars represent the range.

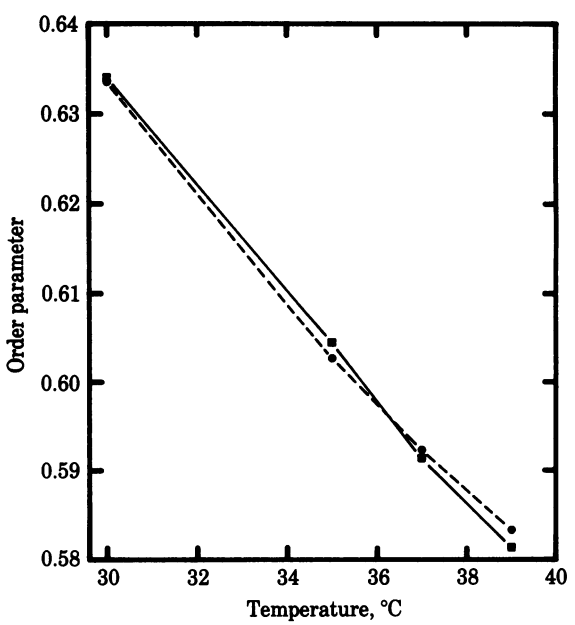

FIG. 2. Change in order parameter with temperature in synaptosomal plasma membranes from LS (●) and SS (■) mice. Samples from a single membrane preparation from each strain were spin-labeled with 5-doxylstearic acid and scanned at the indicated temperatures.

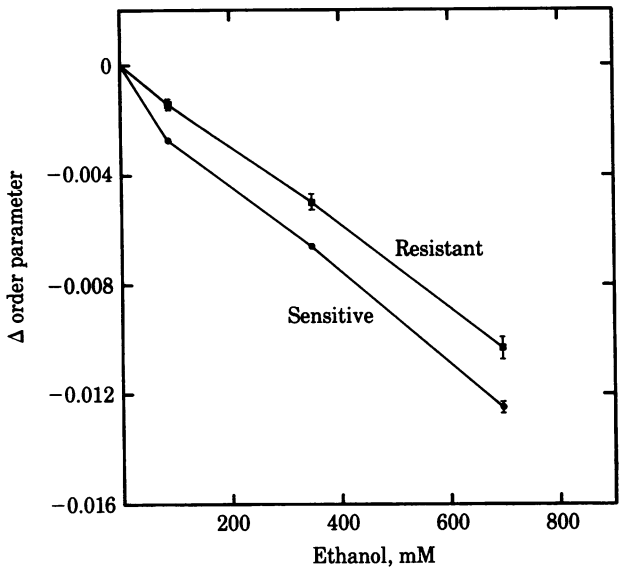

FIG. 3. Change in order parameter on addition of ethanol to synaptosomal plasma membrane from selected sensitive (●) and resistant (■) HS mice. Points are means of nine preparations from individual mice; error bars represent SEM.

An earlier experiment of the same design also gave statistically significant differences in ethanol-induced disordering between the groups ($P < 0.05$) and the ethanol concentrations ($P < 0.001$), with no difference in base-line order parameters.

DISCUSSION

These data show that the ethanol sensitivity of mouse membranes correlates with the sensitivity of the intact mice. The LS and SS lines differ in ethanol sleep time and in the ED_{50} for loss of righting reflex after ethanol injections (13) but do not differ in response to chemically unrelated drugs such as barbiturates (14). This indicates that ethanol has a genetically modifiable site of action that is not shared by barbiturates. Our data (Figs. 1 and 2) show that membranes from the two lines respond differently to ethanol but similarly to changes in temperature, again indicating some degree of specificity for ethanol.

The LS and SS lines were developed by a large selective breeding program, using many pairs of mice in each generation so as to avoid mating of close relatives. Because care was taken to avoid inbreeding, there should be no fortuitously associated biochemical differences, and the observed line difference in membrane responsiveness should represent a direct relationship between membrane disorder and intoxication. Nevertheless, it seemed prudent to check the observation by a test of a different sort, using a heterogeneous population from which we could draw mice that differ in sensitivity without any intervening breeding program. Furthermore, it was advantageous to use a test of sensitivity that could not be confounded by any differences in the rate of ethanol elimination that might exist within the HS population. Therefore, we tested brain ethanol concentrations at a behavioral end point—recovery from ataxia—rather than using sleep time as an index of sensitivity to ethanol. This study of HS mice confirmed the genetic correlation that had been indicated by the LS and SS mice.

With respect to membrane disordering, the lines that had been selectively bred over many generations were remarkably similar to the mice that were selected for sensitivity or resistance to ethanol in the foundation population. When the breeding program was initiated, the HS mice chosen as breeders in the first generation were the longest and shortest sleepers after a standard dose of ethanol and were therefore quite comparable to our selected sensitive and resistant HS mice. This suggests that the initial breeding stock for the two lines already differed in membrane sensitivity to ethanol; subsequent generations apparently accumulated genes for other mechanisms of differential sensitivity, rather than further change in membrane disordering, to produce the slow divergence of the sleep times of the two lines. The differential sensitivity of the synaptosomal plasma membranes of the two lines does not appear to have increased during the selective breeding.

We found no difference in base-line order parameter between the selectively bred lines or between the sensitive and resistant groups of HS mice when they were tested with 5-doxylstearic acid as spin label. Waring *et al.* (15), using liver mitochondria from rats chronically treated with ethanol, found that measurements at 15°C revealed a difference in base-line order parameter that was not apparent at 37°C. We did not see any difference in order parameter between membranes of LS and SS mice at temperatures between 30 and 39°C (Fig. 2). We have recently found that an increased order parameter can be detected in membranes from mice treated chronically with ethanol if 12-doxylstearic acid is used as spin label rather than 5-doxylstearic acid as used here (16). The 12-doxyl probe monitors molecular motion deeper in the membrane than does the 5-doxyl spin label. Thus, it is possible that a genetically determined difference in base-line order parameter might be observable with another probe.

The findings, together with our previous data on pharmacological correlations and on the ethanol resistance of membranes from tolerant mice, suggest that membrane disordering may be the primary mechanism of ethanol intoxication.

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