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Changes in Synaptic Membrane Order Associated with Chronic Ethanol Treatment in Mice

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SUMMARY

The fluidity of brain synaptosomal plasma membranes (SPM) from ethanol-treated mice and control mice was investigated by a sensitive EPR technique. Mice were made tolerant to and physically dependent on ethanol by 3 days of continuous exposure to ethanol vapor. Daily injections of pyrazole were used to stabilize the blood alcohol levels. At the time of withdrawal, SPM were prepared and spin-labeled with 5-, 12-, or 16-doxylstearic acid. "Baseline" order parameters were measured in the absence of added alcohol. The chronic ethanol treatment produced a significant increase in the baseline order parameter that could be detected with the 12-doxyl probe. This indicates a more rigid membrane associated with the withdrawal hyperexcitability. Baseline order parameters measured with the 5-doxyl probe (near the bilayer surface) or with the 16-doxyl probe (near the bilayer interior) were not affected by the chronic ethanol treatment. When ethanol or *t*-butanol was added *in vitro* to membranes labeled with the 5- or 12-doxyl probe, a membrane component of tolerance was revealed; that is, the SPM from ethanol-treated mice required more alcohol to reduce the membrane to a given disordered state. The SPM content of cholesterol and phospholipid was not affected by the chronic ethanol treatment.

INTRODUCTION

Like other anesthetic agents, ethanol appears to act nonspecifically within the hydrophobic regions of neuronal membranes (1, 2). The physical disordering of synaptic membranes by sublethal concentrations of ethanol (11–20 mM) has been demonstrated by Chin and Goldstein (3), using EPR techniques, and by Harris and Schroeder (4), using fluorescence polarization. Membrane-disordering properties are characteristic of short-chain alcohols (1) and may be essential for their *in vivo* action. The hypnotic potencies of short-chain alcohols correlate with their ability to disrupt the structure of neuronal membranes (5).

The development of alcohol tolerance and dependence may be the result of membrane adaptations (6). To compensate for the disordering effects of a resident alcohol, a neuronal membrane might adapt by restoring its order. A reduction in the disordering effect of the alcohol would be indicative of a tolerant state. On removal of the alcohol, the membrane would be left out of balance in a rigid, dependent state.

To test this hypothesis we have measured the order of SPM[2] from alcohol-tolerant/-dependent mice by EPR techniques. If the membranes have adapted to the presence of alcohol during the chronic treatment, their membrane order might be higher than that of controls in the absence of alcohol (baseline order parameter) and in the presence of alcohol added *in vitro*.

In a previous study (7), DBA mice were made tolerant to and dependent upon ethanol by an 8-day liquid diet treatment. The chronic ethanol treatment did not affect the baseline order parameter of SPM spin-labeled with 5-doxylstearic acid, which probes the bilayer surface. However, a membrane component of tolerance was found. That is, more ethanol was required *in vitro* to reduce the membrane order to a given disordered state in SPM from ethanol-treated mice than in SPM from control mice.

Here we confirm these results, using a different mouse strain and mode of ethanol administration, namely the 3-day ethanol inhalation technique of Goldstein (8), which provides a continuous, uniform drug exposure and a precise commencement of drug withdrawal. We used the 5-, 12-, and 16-doxylstearic acid spin labels that explore different depths of the membrane bilayer, because these probes vary in their sensitivity to ethanol and to changes in the composition of lipid bilayers (9), which may be expected after chronic alcohol exposure (10). Only the 12-doxyl label detected an increase in the order of synaptic membranes from ethanol-treated mice, indicating that the membrane changes associated with

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[2] The abbreviations used are: SPM, synaptosomal plasma membranes; BAC, blood alcohol concentrations.

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chronic ethanol exposure were localized within the bilayer.

METHODS

Materials. Ethanol was purchased from U. S. Industrial (New York, N.Y.), and *t*-butanol and pyrazole were obtained from J. T. Baker (Phillipsburg, N.J.). All were reagent-grade and were used without further purification. The spin labels, 5-, 12-, and 16-doxylstearic acids (*N*-oxyl-4′-4′-dimethyloxazolidine derivatives of 5-, 12-, and 16-ketostearic acids), were purchased from Syva (Palo Alto, Calif.).

Alcohol exposure. Male Swiss-Webster mice (8–10 weeks old; Charles River Breeding Laboratories, Wilmington, Mass.) were exposed to ethanol vapor continuously for a period of 3 days as described by Goldstein (8). The mice were given a priming dose of ethanol (1.67 g/kg) with pyrazole (68 mg/kg) i.p. and housed in a chamber containing ethanol vapor at a concentration (7.6–9.3 mg/liter) necessary to produce blood alcohol concentrations of 2.0–2.5 mg/ml. The blood levels were stabilized by daily injections of pyrazole (68 mg/kg i.p.). No pyrazole was given on the day of withdrawal. Daily samples of 0.5 ml of air were taken at the inlet and through the ports in the vapor chamber wall and were assayed enzymatically (11). The alcohol levels in the blood were determined by the Lundquist (12) enzymatic assay. Tail blood samples (10 μl) were taken daily from all mice in experiments where SPM were prepared from single brains or from one-third of the mice when membrane preparations from pooled brains were used. The mean BAC were calculated by adding one-half of the BAC for day 0 (0.5 × 2.0 mg/ml, estimated from the priming dose), the BAC for day 1, the BAC for day 2, and one-half of the BAC for day 3 and dividing this sum by 3.

Control mice. Pyrazole controls and saline controls received daily i.p. injections of pyrazole (68 mg/kg) and 0.9% saline, respectively. Control mice were housed in an air chamber without ethanol. Tail blood samples were taken to control for stress induced by blood sampling.

Membrane preparations. Whole brains were excised from the ethanol-treated and control mice immediately following withdrawal from ethanol. SPM were prepared from individual brains or pooled brain homogenates by the combined flotation-sedimentation density gradient centrifugation technique of Jones and Matus (13). After removal from the sucrose gradient the SPM were washed with isotonic buffer, pelleted by centrifugation at 100,000 × *g* for 20 min, and resuspended in pH 7.0 isotonic buffer (3). The membrane suspensions were stored in liquid nitrogen, then thawed for use and diluted with isotonic buffer to a protein concentration of 20 mg/ml (14). SPM were spin-labeled with 5-, 12-, or 16-doxylstearic acid and prepared for EPR analysis in the absence or presence of alcohol as previously described (5). A final protein concentration of 17 mg/ml was maintained, and the spin label concentrations were calculated to be less than 1% by weight of membrane lipid.

Measurement of order parameters. The spectral data of spin-labeled SPM were collected by a Varian E-104A EPR spectrometer (9.5 GHz) and computer-analyzed as previously described (9). The order parameter was calculated by the method of Hubbell and McConnell (15), with correction of the observed inner hyperfine splittings by the method of Gaffney (16). The reference values for 5-doxylpalmitate were used as a crystalline standard. Each sample was scanned and analyzed three times to produce an average order parameter for that sample. The cavity temperature was regulated to within 0.1°. SPM spin-labeled with 5-doxylstearic acid were measured at 19° and 37°. SPM containing 12-doxyl and 16-doxyl labels were measured at 19° since the high-field outer peak is not well resolved at higher temperatures.

Lipid extraction and analysis. After removal from the sucrose gradient, SPM were washed four times with glass-distilled water, pelleted each time at 100,000 × *g* for 20 min, resuspended in glass-distilled water, and stored in liquid nitrogen. Thawed membranes suspensions were first extracted with methanol (0.5 ml/mg of protein), sonicated, and centrifuged at 18,000 × *g* for 20 min. The supernatant was stored on ice and the pellet was extracted twice with 1 ml of chloroform/methanol (1:1, v/v) per milligram of protein. The three extracts were combined, the solvent ratios were adjusted to make the extract contain 1 volume of water to 20 volumes of chloroform/methanol (2:1, v/v), and the final extract was washed with 0.2 volume of 0.88% KCl according to the method of Folch *et al.* (17). The lower phase was washed with one-sixth volume of chloroform/methanol/0.1 M potassium citrate (3:48:47) to cleave proteins from proteolipids (18), evaporated to dryness, and dissolved in chloroform/methanol (2:1, v/v). Cholesterol content was determined spectrophotometrically by the assay of Rudel and Morris (19) and phospholipid by the assay of Morrison (20).

RESULTS

Baseline order parameters. The intrinsic order of spin-labeled SPM was measured at 19° ± 0.1° with the 5-, 12-, and 16-doxylstearic acid probes. Membranes were prepared from individual brains. These particular mice ($N = 11$) had daily BAC ranging from 1.65 to 2.79 mg/ml, with a 3-day mean BAC of 2.21 mg/ml, which is sufficient to produce physical dependence (8). The mean (± standard error) weight loss for these ethanol-treated mice was 7.2 ± 0.8% as compared with a weight gain of 1.5 ± 1.4% for control mice. Figure 1 shows the percentage increase in baseline order parameter due to chronic ethanol treatment as observed for each spin label. A significant increase of 1.2% was observed with the 12-doxyl probe. The absolute values (mean ± standard error) for SPM from ethanol-treated and control mice, respectively, were 0.704 ± 0.0017 and 0.704 ± 0.0018 (5-doxyl, not significant, $N = 5$ pairs); 0.517 ± 0.0008 and 0.511 ± 0.0010 (12-doxyl, $p < 0.01$, $N = 6$ pairs); and 0.228 ± 0.0005 and 0.227 ± 0.0010 (16-doxyl, not significant, $N = 3$ pairs). Apparently the ethanol-treated mice have increased membrane order in the region probed by 12-doxyl, but not by 5-doxyl or 16-doxylstearic acid. Also, when measured with the 5-doxyl label at 37.0° ± 0.1° the baseline order parameters of SPM from ethanol-treated and control mice were not significantly different (0.591 ± 0.0010 and 0.591 ± 0.0003, $N = 3$ pairs).

Disordering by alcohols. Ethanol or *t*-butanol was

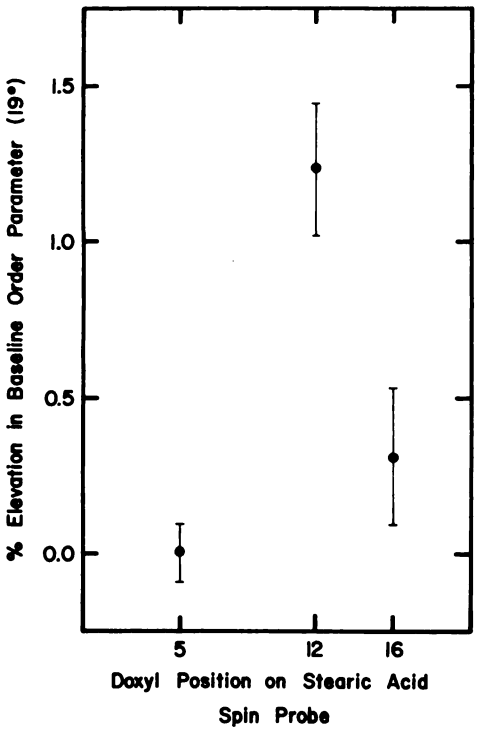

FIG. 1. *Effect of chronic ethanol treatment by inhalation on synaptic membrane order in the absence of added alcohol*

The percentage increase in baseline order parameter for SPM from ethanol-treated mice compared with pyrazole control mice was measured at 19° using the 5-, 12-, and 16-doxylstearic acid spin labels. The points represent means ($N \geq 3$) and the vertical bars represent standard error.

added *in vitro* to spin-labeled SPM from ethanol-treated and pyrazole control mice. Membranes were prepared from pooled brains. The BAC for these mice ranged from 1.76 to 3.43 mg/ml with a 3-day mean BAC of 2.30 mg/ml. The mean (± standard error) weight loss for these ethanol-treated mice ($N = 27$) was 9.7 ± 0.6% as compared with a weight loss of 2.6 ± 0.7% for control mice ($N = 27$). The disordering effect was observed by measuring membrane order at different alcohol concentrations with 5-doxylstearic acid at 37° and 12-doxylstearic acid at 19°.

No difference in the baseline order was observed with the 5-doxyl probe, but the effect of chronic ethanol treatment on the membrane order was revealed when alcohol was added *in vitro* (Fig. 2). SPM from ethanol-treated mice, designated "tolerant membranes," required a higher alcohol concentration than controls to reduce the membrane order to a given disordered state. The ethanol concentration required to reduce membranes to an order parameter (S) of 0.582, for example, was 695 mM for tolerant membranes and 520 mM for controls (Fig. 2, left). Similarly tolerant membranes required 230 mM *t*-butanol and controls required 180 mM to reach $S = 0.582$ (Fig. 2, right). This is an example of membrane cross-tolerance. The significance of this difference between tolerant and control membranes was demonstrated by a two-way analysis of variance for the *in vitro* addition of ethanol [$F(1,24) = 11.0$; $p < 0.01$] and for the *in vitro* addition of *t*-butanol [$F(1,18) = 8.57$; $p < 0.01$].

In the region of the bilayer probed by the 12-doxyl label, tolerant membranes were more rigid than control membranes in the absence and presence of alcohol (Fig. 3). A two-way analysis of variance showed a significant difference between tolerant and control membranes either with ethanol [$F(1,40) = 62.4$; $p < 0.01$] or with *t*-butanol [$F(1,24) = 12.9$; $p < 0.01$]. This is also an example of membrane tolerance and cross-tolerance. To reduce S to 0.500 in tolerant membranes, 515 mM ethanol or 200 mM *t*-butanol was required as compared with 320 mM ethanol or 145 mM *t*-butanol for control membranes.

Cholesterol and phospholipid content. No differences in cholesterol content, phospholipid content, or the cholesterol/phospholipid molar ratio could be detected between membranes from ethanol-treated mice and controls (Table 1). The variability in cholesterol and phosphorus assays was due to variations in recovery of protein, but the ratio of cholesterol to phospholipid in individual brains was relatively constant.

Pyrazole controls. Pyrazole treatment alone did not affect the membranes. The order of SPM from mice receiving the 3-day pyrazole treatment (pyrazole controls) was compared with that of SPM from mice receiving a 3-day saline treatment (saline controls). As measured with the 5-doxyl probe at 37° or the 12-doxyl probe at 19°, the mean order parameters for SPM from pyrazole controls were identical with those of saline controls in the absence of ethanol (0.591 for 5-doxyl, 0.511 for 12-doxyl) or in the presence of 350 mM ethanol (0.585 for 5-doxyl, 0.500 for 12-doxyl).

DISCUSSION

Behavioral responses to chronic alcohol treatment include (a) intoxication following initial exposure, (b) recovery from the effects of intoxication during continuous exposure (development of tolerance), and (c) hyperexcitability following withdrawal (physical dependence). If these stages represent adaptations of brain membranes to ethanol, as has been suggested (6), the following sequential effects might be observed. (a) Intoxication may correspond to the disordered state induced by alcohol *in vitro*. (b) Continuous exposure may trigger an ordering process to offset the presence of alcohol in the membrane. Tolerance may be seen as the requirement for more alcohol *in vitro* to reach a given disordered state. (c) Physical dependence, by the same reasoning, may reflect an ordered state which exists following the removal of the alcohol from the membrane. This study has focused on membrane changes that may be adaptive in nature and are associated with tolerance and physical dependence.

The physical state of synaptic membranes from tolerant/dependent mice was assessed by measuring the membrane order at different depths in the bilayer. A membrane component of dependence, i.e., increased order, was detected by the 12-doxylstearic acid spin probe following chronic alcohol exposure. This apparent adaptation was not detected by the 5-doxyl or the 16-doxyl probe. Probes located at different depths may be differentially sensitive to changes in bilayer structure and composition. The 12-doxyl probe is more sensitive than 5-doxyl to the ordering produced by an increase in the cholesterol (9), and might also be sensitive to changes in the saturation of phospholipid fatty acids, since the ole-

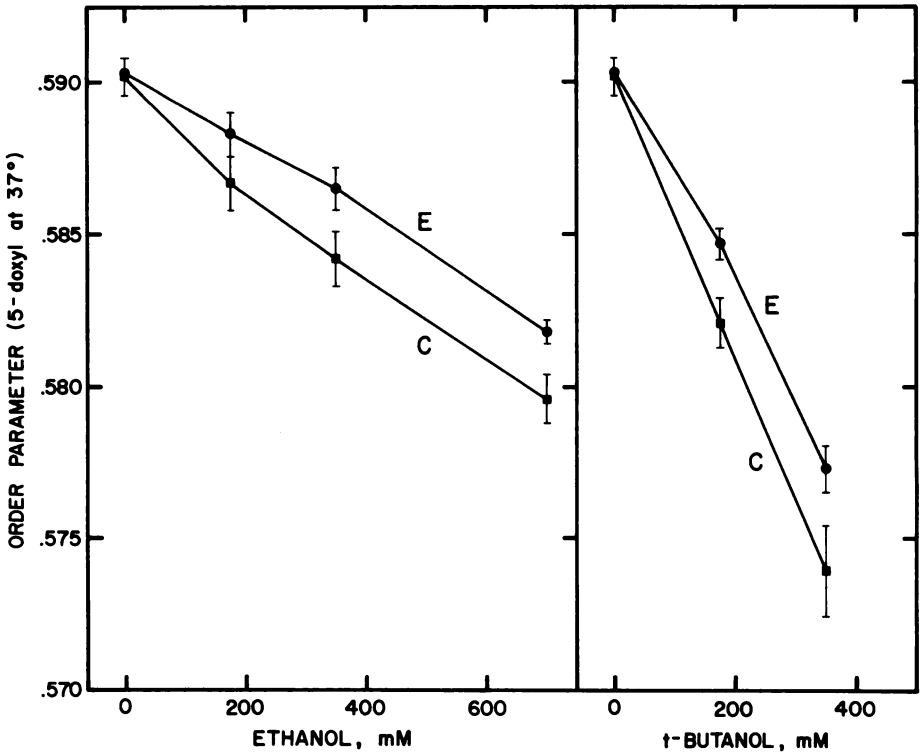

FIG. 2. *Effect of chronic ethanol treatment by inhalation on synaptic membrane order measured with 5-doxylstearic acid at 37° in the presence of alcohol added in vitro*

E, SPM from ethanol-treated mice; *C*, SPM from pyrazole control mice. Left, disordering by ethanol; right, disordering by *t*-butanol. The points represent means ($N = 4$) and the vertical bars represent standard error.

finic positions are located in the central portion of the acyl chains. The doxyl probes also vary in their sensitivity to disordering agents. The rank order of sensitivity to the disordering effect of ethanol or heat (expressed as percentage reduction in order parameter) was 16-doxyl > 12-doxyl > 5-doxyl (9). This selective sensitivity to alcohol disordering with regard to the depth within the bilayer has also been demonstrated by NMR and fluorescence studies. Measuring deuterium NMR quadrupole splittings (order parameter) of specifically deuterated

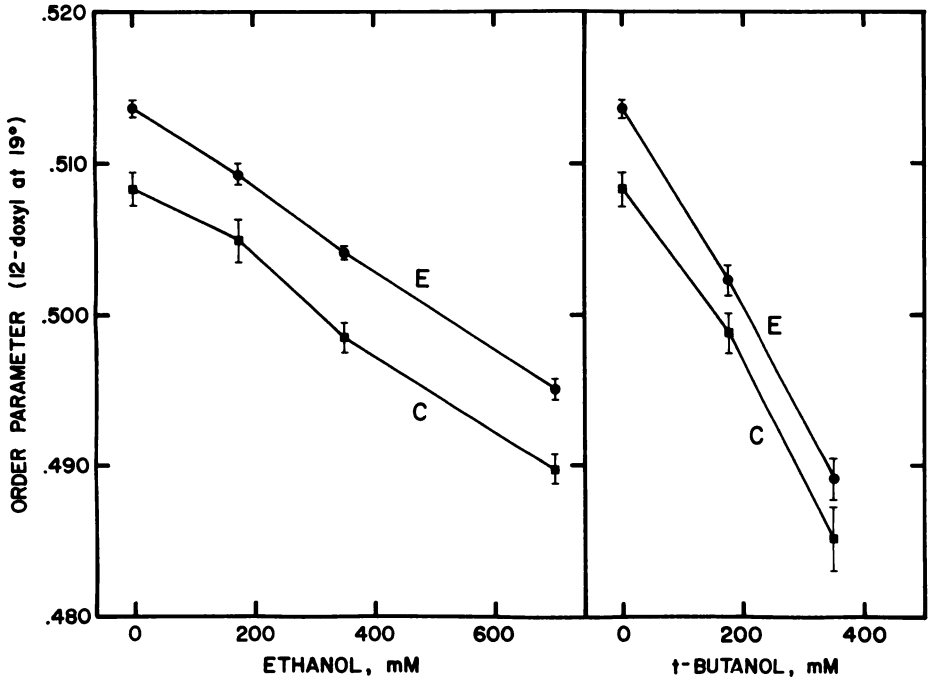

FIG. 3. *Effect of chronic ethanol treatment by inhalation on synaptic membrane order measured with 12-doxylstearic acid at 19° in the presence of alcohol added in vitro*

E, SPM from ethanol-treated mice; *C*, SPM from pyrazole control mice. Left, disordering by ethanol; right, disordering by *t*-butanol. The points represent means ($N \geq 5$) and the vertical bars represent standard error.

TABLE 1

Effects of chronic ethanol treatment on cholesterol and phospholipid contents of synaptic membranes

Each group represents data from 10 individual brains. Each value is the mean ± standard error.

Group	Cholesterol (C)	Phospholipid (P)	C/P molar ratio
	nmoles/mg protein	*nmoles/mg protein*	
Ethanol-treated	467 ± 25	660 ± 32	0.707 ± 0.008
Pyrazole controls	465 ± 14	668 ± 16	0.696 ± 0.008

dimyristoylphosphatidylcholines, Turner and Oldfield (21) observed the largest reduction by benzyl alcohol and the largest enhancement by cholesterol at the C-8, C-10, and C-12 positions. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (a probe of the membrane core) was more sensitive than 1-aminopyrene (a probe of the membrane surface) to the disordering effect of ethanol in synaptic membranes (4). Using this diphenylhexatriene probe, Johnson *et al.* (22) detected an increase in the order of reconstituted synaptic membrane lipid extracts from ethanol-tolerant mice.

No change in baseline membrane order associated with chronic ethanol treatment could be detected by the 5-doxyl probe at either 19° or 37° in this study [or in our previous study, using mice subjected to 8 days of ethanol liquid diet (7)]. Contrary to our results, Rottenberg *et al.* (23) reported an elevated order parameter for 5-doxyl-labeled synaptic membranes from rats administered alcohol by liquid diet for 35 days.

Whether the membranes were spin-labeled with the 5-doxyl or the 12-doxyl probe, the membranes were tolerant to the *in vitro* addition of ethanol and cross-tolerant to *t*-butanol. Compared with control membranes, more alcohol was required to reduce membranes from tolerant/dependent mice to a given disordered state. Similar results have been reported for SPM (7, 23), erythrocytes (7), mitochondrial membranes (23, 24), and reconstituted lipids from synaptic membranes (22).

The meaning of the two types of tolerance represented by Figs. 2 and 3 is not yet clear. Responses to ethanol *in vivo* or *in vitro* usually are directly proportional to ethanol concentration, as would be expected for drugs that act by partition in membranes. The literature contains many examples of ethanol tolerance data in the form of parallel dose responses shifted along a linear abscissa. The parallel shift in the curves of Fig. 3 (12-doxyl) can be described as an increase of about 200 mM ethanol to bring the order parameter to the same value as controls. By contrast, with the 5-doxyl label, the baseline order parameter seems unchanged by the chronic ethanol treatment and there is an evident tendency for the lines to diverge. Most of the separation occurs at low ethanol concentrations, and the slopes of the lines in Fig. 2 are not significantly different. With the observed amount of variability, it is not clear whether the lines are straight; if they are straight and diverge from a single point, then one cannot express the tolerance in millimolar units but as a percentage increase in ethanol concentration to achieve the original effect. Both the divergent and the parallel types of *in vitro* changes have recently been reported by Waring *et al.* (25) in liver mitochondrial membranes from rats treated chronically with ethanol.

In our earlier study (10), the SPM of mice treated for 8 days with ethanol in a liquid diet contained more cholesterol than controls and were resistant to ethanol *in vitro*. It seemed reasonable that the increased cholesterol might be an adaptive response to the disordering effect of ethanol because cholesterol effectively orders biomembranes (26) and lipid bilayers (27). However, Johnson *et al.* (22) reported that changes in membrane cholesterol could not account for resistance to ethanol in membranes from chronically treated mice. We found no change in membrane cholesterol or phospholipids in the present study, despite evident changes in membrane order and response to ethanol *in vitro*. Another adaptive mechanism is the incorporation of more saturated fatty acids into membrane phospholipids (28). This was detected in brain synaptic membranes from TO Swiss mice administered alcohol by inhalation (29). Reductions in arachidonic (20:4) and docosahexenoic (22:6) acids with an increase in stearic acid (18:0) occurred during a 10-day treatment; most of this change occurred during the first 2 hr.

Changes in membrane lipid composition may depend on the mode of ethanol administration. By inhalation, mice are continuously exposed to a uniform concentration of alcohol. The blood alcohol concentration is boosted within minutes to 2 mg/ml by the initial priming injection and maintained at a constant level for the entire 3 days by pyrazole, which inhibits liver alcohol dehydrogenase competitively with ethanol. In contrast, by the liquid diet technique, the continuity of alcohol intake and the blood alcohol concentration is dependent on individual eating and drinking patterns. The mean blood alcohol concentration of the group starts at zero and rises steadily for the duration of the ethanol administration but that of individual mice may drop to zero at times (30). The mean body weight loss for an 8-day liquid diet with DBA mice was 28% (30) compared with less than 10% for the 3-day inhalation ethanol-treated mice.

In conclusion, we can detect an increase in synaptic membrane order associated with chronic ethanol exposure if a suitable probe is employed. This increase in membrane rigidity may be a membrane component of dependence. Changes in brain synaptic membrane cholesterol were not responsible for these adaptations.

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