MOLECULAR PHARMACOLOGY, 25:401–409

Physical Properties and Lipid Composition of Brain Membranes from Ethanol Tolerant-Dependent Mice

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Received August 17, 1983; Accepted January 11, 1984

SUMMARY

DBA/2 mice were made tolerant to and dependent on ethanol by administration of an ethanol-containing liquid diet for 7 days. Fluorescent probe molecules were used to estimate the fluidity and ethanol sensitivity of brain synaptic membranes from these mice. The fluorescence polarization of *cis*-parinarate, *trans*-parinarate, and 1,6-diphenyl-1,3,5-hexatriene (probes of the membrane core) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (a probe of the membrane surface) was higher in membranes from ethanol tolerant-dependent mice than in membranes from control mice. The decrease in fluorescence polarization produced by *in vitro* exposure to ethanol was attenuated in membranes from ethanol tolerant-dependent mice when 1,6-diphenyl-1,3,5-hexatriene was used as the probe, but not when 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene was used. These results indicate that chronic ingestion of ethanol decreased the fluidity and the ethanol sensitivity of the synaptic membranes. In contrast to the alterations observed with intact membranes, liposomes of lipids extracted from synaptic membranes of control and ethanol tolerant-dependent mice did not differ in their physical properties. Analysis of membrane lipids demonstrated that chronic ethanol treatment selectively decreased the unsaturated acyl groups of phosphatidylserine without altering the acyl composition of other phospholipids or sphingolipids. The amount of each phospholipid was not changed, but membrane cholesterol was decreased by chronic ethanol ingestion. Use of 2-dimensional thin-layer chromatography allowed the quantitation of 10 different gangliosides. The concentrations of these lipids were unchanged in synaptic membranes from ethanol tolerant-dependent mice. Thus, the changes in membrane physical properties produced by chronic ingestion of ethanol may be due, at least in part, to altered acyl composition of phosphatidylserine. The differences observed between intact membranes and extracted lipids suggest, however, that chronic ethanol treatment also produced changes in the lipid arrangement or lipid-protein interactions of the intact membranes.

INTRODUCTION

Recent investigations of alcohol intoxication, tolerance, and dependence have focused on cell membranes (1–3). Information obtained from electron paramagnetic resonance (EPR) and fluorescence polarization studies indicate that *in vitro* exposure to ethanol disorders the lipid portions of brain (2–5) and liver membranes (6, 7). Furthermore, the chronic ingestion of ethanol induces changes in the physical properties of cell membranes. Brain and liver membranes from rodents treated chronically with ethanol are resistant to the membrane-disordering effects of ethanol measured *in vitro* (2, 3, 6, 7). In addition, some (3, 6, 7), but not all (2), studies report increased membrane rigidity (measured in the absence of ethanol) after chronic ethanol treatment. A recent study by Lyon and Goldstein (3) indicates that the increased rigidity is restricted to the middle portions of the acyl groups. Such changes in membrane order and drug sensitivity are suggestive of homeoviscous adaptation and represent a plausible mechanism for tolerance and dependence (1, 8). Membrane components responsible for the alterations in physical properties are not known. Most of the studies cited above used intact mem-

This work was supported by funds from the Veterans Administration and by United States Public Health Service Grants DA02855, AA06399, and MH-37377.

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0026-895X/84/030401-09$02.00/0

branes, but one series of experiments found changes in alcohol sensitivity with lipids extracted from brain membranes of control and alcohol-treated animals (9–11), suggesting an alteration in membrane lipid composition. Cholesterol content and phospholipid acyl group saturation are important determinants of membrane physical properties and represent potential sites for adaptation to alcohol. Chronic alcohol exposure increases membrane cholesterol under some (12, 13), but not all (3), conditions. However, these changes do not appear sufficient to explain the changes in membrane physical properties (3, 9). Alterations in the acyl composition of brain phospholipids have been noted following chronic ethanol administration (14–16), but these changes have not been detected in all studies (13, 17, 18). These discrepancies may be due to the analysis of the total membrane acyl groups in some studies (13, 14, 17, 18) and the acyl composition of individual phospholipids in others (15, 16) or to the use of membrane preparations containing myelin and mitochondria in addition to plasma membranes. Gangliosides represent a major lipid component of the synaptic membrane, and studies of model membranes indicate that gangliosides alter the physical properties and the ethanol sensitivity of lipid vesicles. These findings suggest that changes in brain gangliosides might be relevant to the membrane effects of ethanol (19).

The observations presented above raise a number of questions regarding the effects of chronic ethanol ingestion on brain membranes. Our previous studies demonstrated that fluorescent probes are sensitive indicators of the acute effects of alcohols (and barbiturates) and can be used to show changes in ethanol sensitivity at different membrane depths, in different membrane fractions, and in different membrane lipids (4, 5). One goal of the present study was to use fluorescent probes to determine which regions of brain membranes change in regard to baseline order and ethanol sensitivity as a result of chronic ethanol treatment. Another objective was to compare intact membranes with extracted lipids to determine whether changes in lipid composition alone could be sufficient to account for the changes in intact membranes. After ensuring that our regimen of ethanol ingestion produced tolerance and dependence *in vivo*, and that this treatment altered the physical properties of synaptic membranes, we carried out a detailed analysis of the lipid composition of these membranes. In line with the current "state of the art," we felt that the most definitive analysis should use SPM[5] and measure the content of individual phospholipids, sphingolipids and cholesterol, and the acyl composition of gangliosides and each phospholipid. This report presents the results of such an analysis.

METHODS

Chronic ethanol treatment. Male DBA/2 mice were obtained from Simonsen Laboratories (Gilroy, Calif.) and weighed 19–25 g at the beginning of the experiments. They were housed singly and given free access to tap water at all times. Liquid diet was delivered in Richter tubes, which were washed and filled each day. Mice were given Slender liquid diet (chocolate flavor, Carnation Company, Los Angeles, Calif.) ad libitum for 3 days. This acclimation to the unadulterated diet led to more consistent consumption of the ethanol-containing diet. Following the 3-day acclimation, a diet containing 88% (v/v) Slender, 5% (v/v) ethanol, and 7% (v/v) water was given for 7 days. This diet provided 29% of the calories as ethanol. The control group was pair-fed an equicaloric diet with sucrose substituted for ethanol. The apparent consumption of ethanol was 14–16 g/kg/day for the first 2 days and reached 20–22 g/kg/day during the last 2 days. This consumption is termed "apparent" because we found that ethanol evaporates from the surface of the diet, reducing the concentration of ethanol in the region accessible to the mouse. During a 24-hr period, this decrease can amount to 40%. The ethanol concentration in the neck and body of the tube does not change appreciably, and the surface concentration depends on how much and how often the animal drinks and whether mixing occurs. Thus, consumption of ethanol could not be determined precisely, but we estimate that it was about 20% less than the values given above. During the period of alcohol ingestion, mice in both the control and ethanol groups lost about 15% of their body weight. Animals showing excessive weight loss (>20%) or low apparent consumption of ethanol (<10 g/kg/day) during the last 3 days were not used in the experiments.

Measurement of tolerance and dependence. After 7 days of exposure to the ethanol- or sucrose-containing diets, the solutions were removed, and the rectal temperature and convulsions on handling were monitored (20) at 7 hr after withdrawal. Tolerance to ethanol was determined 7–8 hr after withdrawal of ethanol by measuring either the ethanol ED_{50} for loss of righting reflex (20) or the brain ethanol concentration at the time of regaining of the righting reflex following administration of ethanol (4 g/kg). Ethanol was injected i.p. as a 20% (w/v) solution in 0.9% NaCl. For determination of brain ethanol, the brain was removed, weighed, and homogenized in water containing 0.01% 1-propranol (internal standard). Protein was precipitated with 0.1 N perchloric acid. An aliquot of the supernatant was analyzed by gas chromatography on Porapak Q, 80–100 mesh, at 190°.

Preparation of SPM. Mice fed ethanol-containing or isocaloric control diets were killed (without withdrawal except as noted), and whole brain SPM (SPM-1 plus SPM-2) were prepared by Ficoll and sucrose density centrifugation as described previously (5, 21). Membranes were suspended in PBS (NaCl, 8 g/liter; KCl, 0.2 g/liter; 0.2 g/liter; KH_2PO_4, 0.2 g/liter; $Na_2HPO_4 \cdot 7H_2O$, 1.15 g/liter; Hepes = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.48 g/liter; pH 7.4) at a concentration of 1–3 mg of protein per milliliter and kept at 4° before analysis.

Lipid extraction. To prepare a "total" lipid extract, SPM were pelleted and the supernatant was removed as completely as possible. The pellet was suspended in methanol and mixed with an equal volume of chloroform under nitrogen. After vortexing, the solution was kept at −20° for 1–2 hr and then centrifuged. The resulting pellet was extracted with chloroform/methanol (2:1) and centrifuged. The two supernatants were combined to give a "total" lipid extract which was stored under argon at −20°. This extraction procedure removes all membrane lipids. This was verified by showing that repeated extraction of the pellet yielded no additional lipid and by showing that extraction of radiolabeled membranes removed >99.5% of the phospholipid.[6] Gangliosides were removed from the lipid fraction by treating the total lipid extract with 0.2 volume of 100 mM KCl. After centrifugation, the organic phase was washed twice with chloroform/methanol/100 mM KCl (3:48:47) and once with water (22). The washed organic phase was dried under nitrogen, resuspended in chloroform/methanol (1:1), and stored under argon at −20°.

Fluorescence measurements. An HH-1 T-format polarization spectrofluorimeter (BHL Associates, Burlingame, Calif.) with fixed excitation and emission polarization filters was used to measure fluorescence intensity parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the polarization phase

[5] The abbreviations used are: SPM, synaptic plasma membranes; PBS, phosphate-buffered saline; DPH, 1,6-Diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; THF, tetrahydrofuran.

[6] R. J. Hitzemann, unpublished data.

of the exciting light (5). Polarization of fluorescence $[(I_\parallel - I_\perp)/(I_\parallel + I_\perp)$ and intensity of fluorescence $(I_\parallel + 2I_\perp)$ were calculated by an on-line microprocessor. For DPH and TMA-DPH, the excitation wavelength was 362 nm, and a 03FCG001 filter (Melles Griot, Irvine, Calif.) was used in the excitation beam and 003FIR045 filters were used for the emitted light. In some experiments, KV389 filters (Shott Optical, Duryea, Pa.) were used for the emission. The use of these rather than the 003FIR045 filters allowed the probe concentration to be reduced by 10-fold. Equivalent results were obtained with both filters and probe concentrations. For *cis*- and *trans*-parinarate, the excitation wavelength was 324 nm, and 03FIV113 or KV389 filters were used for the emission. No excitation filter was used. Cuvette temperature was maintained by a circulating water bath and monitored continuously by a thermocouple inserted into the cuvette to a level just above the light beam.

SPM were diluted to 50 μg of protein per milliliter of PBS, and fluorescent probes were incorporated at 35° for 30 min with frequent vortexing (5). Vesicles were prepared from extracted lipids as described previously (4). Probes were dissolved in THF (DPH), THF-water, 1:1 (TMA-DPH), or ethanol (parinarates) and were added in a volume of 0.3–0.5 μl/ml to give a probe concentration of 0.3–0.5 μg/ml. The fluorescence of parinarates was reduced by exposure to air, and all solutions containing this probe were kept under argon. Probes were purchases from Molecular Probes, Inc. (Junction City, Ore.). After incorporation of probe, samples were placed in the fluorimeter and maintained at 35°. Control levels of fluorescence (baseline) were determined, and an aliquot of ethanol solution was added to the cuvette; fluorescence was determined 3–5 min later. The temperature dependence of fluorescence was studied by placing the sample in the thermostated compartment at 35°, then reducing the temperature at a rate of 0.5°/min with a refrigerating water bath. The suspension was stirred continuously, and cuvette temperature and fluorescence were recorded at 1-min intervals. To minimize any systematic artifacts, membranes from control and ethanol-treated animals were tested in an alternating order.

Analysis of membrane lipids. The total membrane lipid extract was analyzed as described in detail elsewhere (23, 24). Briefly stated, the individual phospholipids were separated by 2-dimensional thin-layer chromatography (25). Plates were run in duplicate; one plate was used for the analysis of phospholipid P and the other plate was used for the analysis of phospholipid acyl group composition (23). Gangliosides were isolated from the total lipid extract and analyzed essentially as described by Ledeen and Wu (26). Ganglioside levels were measured using the resorcinol technique (27).

Other analyses. Protein was determined by the method of Lowry *et al.* (28). Concentration-response curves and temperature-response curves for membranes from control and ethanol-treated animals were compared by an analysis of variance for repeated measures. For comparison of baseline polarization values, membranes from control and ethanol-treated animals which were prepared on the same day and analyzed at the same time were paired, and differences between these pairs were evaluated by Student's *t*-test for paired samples. The ED_{50} values were determined by the method of Dixon (29).

RESULTS

Tolerance and dependence in vivo. The liquid diet technique of ethanol administration has been shown to produce tolerance and dependence in mice (30, 31). However, it was important to quantitate the degree of tolerance and dependence achieved with the particular strain of mice and with the experimental protocol used in the present studies. Withdrawal of the ethanol diet resulted in tremors, rigidity, convulsions on handling, and spontaneous seizures as described by others for this strain of mice (32). At 7 hr after withdrawal, a maximal seizure score was obtained (Table 1) and was accompanied by hypothermia ($\Delta = -3.1°$ compared with the control group) as reported previously (20, 30). Two measures of tolerance, the brain ethanol concentration at the time of regaining righting reflex and the ethanol ED_{50} for loss of righting reflex were higher in the chronic ethanol group as compared to the control group (Table 1).

TABLE 1

In vivo measurement of ethanol tolerance and dependence

DBA/2 mice were fed an ethanol-containing liquid diet or an isocaloric control diet for 7 days. All measurements were made 7–8 hr after withdrawal of the diets.

Parameter measured	Control	Chronic ethanol
Seizure score[a]	0.2 ± 0.1	3.1 ± 0.8[b]
Brain ethanol concentration[c] (regaining righting reflex) (mg/g wet wt.)	4.1 ± 0.1	4.8 ± 0.1[b]
Ethanol ED_{50} for loss of righting reflex[d] (g/kg)	2.1 (1.8–2.4)	3.6 (3.1–4.2)[e]

[a] "Convulsions on handling" were scored by the method of Goldstein (20, 32). Values are means ± standard error of the mean, $n = 9$.

[b] Significantly different from control group ($p < 0.001$).

[c] Measured at the time of regaining of righting reflex. Loss of righting reflex was produced by ethanol. Values are means ± standard error of the mean, $n = 5$ and 9.

[d] Values in parentheses are 95% confidence limits.

[e] Significantly different from control group ($p < 0.05$).

Chronic ethanol treatment and the ridigity of synaptic membranes. The fluidity or order[7] of brain membranes was evaluated by the fluorescence polarization of several probe molecules. The fluorescence polarization of TMA-DPH, DPH, *cis*-parinarate, and *trans*-parinarate was higher for SPM from ethanol tolerant-dependent mice as compared with pair-fed controls (Fig. 1). The last three molecules are probes of the lower (methyl terminal) portions of the lipid acyl groups (34, 35), whereas TMA-DPH is a probe of the glycerol backbone regions and upper (carboxyl) portions of the acyl groups of the membrane (36). Thus, these results indicate an increased rigidity at all membrane depths following chronic alcohol treatment. This difference was seen when membranes from both groups were assayed at the same temperature (35°). However, ethanol withdrawal produces hypothermia, which further increases the rigidity of brain membranes. Thus, the membrane differences between control and alcohol-dependent mice may be greater *in vivo* than *in vitro*. Comparison of the different probes in control membranes indicated that the fluorescence polarization of TMA-DPH was higher than that of DPH and the polarization of *trans*-parinarate was higher than that of *cis*-parinarate. These results are consistent with those of others and have been interpreted to indicate that the membrane surface is more rigid than the core (TMA-DPH versus DPH) and that *trans*-parinarate partitions more readily than *cis*-parinarate into rigid domains of the membrane (34–36). The temperature dependence of

[7] Recent analyses indicate that the steady state polarization of DPH is related to the packing of membrane lipids but not necessarily related to the microviscosity of lipids (33, 34). In the present report, we use the terms "fluidity" (or "rigidity") and "order" to indicate the membrane physical properties (e.g. lipid packing) that are reflected in the fluorescence polarization of the probe molecules.

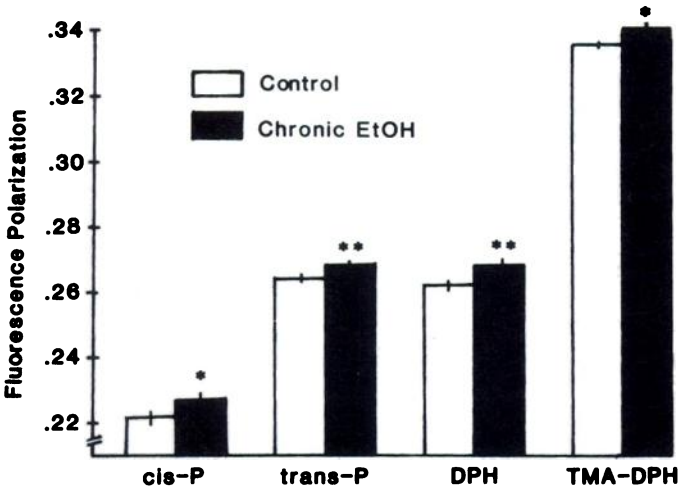

FIG. 1. *Fluorescence polarization of probes incorporated into synaptic membranes from control (open bars) or ethanol tolerant-dependent (filled bars) mice*

From left to right, the pairs of bars represent data obtained with *cis*-parinarate, *trans*-parinarate, DPH, and TMA-DPH. Assay temperature was 35°. Values are the means ± standard error of the mean from 9–12 different membrane preparations. *, Significantly different from control ($p < 0.05$); **, $p < 0.01$.

the fluorescence polarization of DPH was determined using SPM from control and ethanol tolerant-dependent mice. The response of the membranes to temperature was similar for the two groups, with the membranes from the ethanol-treated mice being less fluid at all temperatures (Fig. 2).

The fluorescence polarization of DPH was also determined in lipids extracted from SPM. In contrast to the differences observed with intact SPM, the polarization of DPH at 35° was not different for a total lipid extract from control (0.253 ± 0.002) or chronic ethanol (0.253 ± 0.001) groups or for a washed lipid extract (control, 0.254 ± 0.002; chronic ethanol, 0.251 ± 0.002) (all values are means ± standard error of the mean; $n = 9$). As noted previously (4, 5) the polarization of DPH is lower in lipid extracts than in intact synaptic membranes. These data and others (34) suggest that proteins increase the order of fluid lipids.

Drug sensitivity of membranes from tolerant-dependent mice. Fluorescent probes were also used to evaluate the effects of chronic ethanol consumption on the sensitivity of synaptic membranes and membrane lipids to the disordering effects of ethanol. SPM from ethanol tolerant-dependent mice were resistant to the effects of ethanol on the polarization of DPH (Fig. 3). Chronic alcohol consumption shifted the concentration-response curve to the right, demonstrating a membrane tolerance of 2- to 3-fold ($F = 5.27$, $df = 1,22$; $p < 0.05$). For brain microsomal and mitochondrial membranes, the tolerance was smaller and less consistent than that observed for SPM (data not shown). SPM from control and ethanol tolerant-dependent mice were also tested with TMA-DPH. TMA-DPH was not as sensitive as DPH to the *in vitro* ethanol exposure and did not detect any difference in the response of control and chronic ethanol SPM (Fig. 4). Similar results were also obtained with *trans*-parinarate (data not shown). Lipids were extracted from the SPM preparations used to obtain the data in Fig. 3, and the ethanol sensitivity of these lipid vesicles was also studied with DPH. The total lipid extract (phospholipids, neutral lipids, and galactolipids) and the washed lipid extract (mainly neutral and phospholipids) were less sensitive than the intact SPM to the membrane-disordering effect of ethanol, in agreement with our earlier report (4). In addition, the lipids from control and ethanol-tolerant mice showed a similar response to ethanol (Fig. 5). Thus, the membrane tolerance observed with intact SPM was not detected with the extracted lipids.

To determine whether the alcohol withdrawal state altered membrane order or ethanol sensitivity, membranes were prepared from mice withdrawn from ethanol for 8 hr (time of maximal withdrawal severity). Results obtained with these SPM were similar to those shown in Figs. 1 and 3 for non-withdrawn mice. Thus, the in-

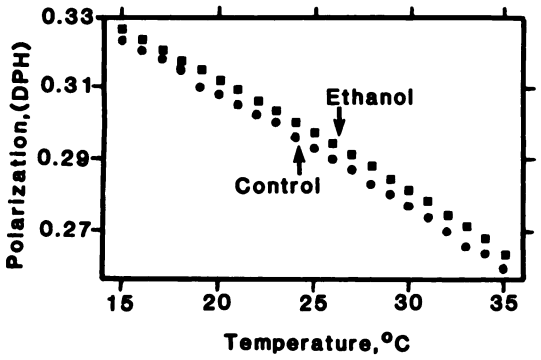

FIG. 2. *Temperature dependence of the fluorescence polarization of DPH in synaptic membranes from control* (●) *and ethanol tolerant-dependent* (■) *mice*

Each point represents the mean from nine membrane preparations.

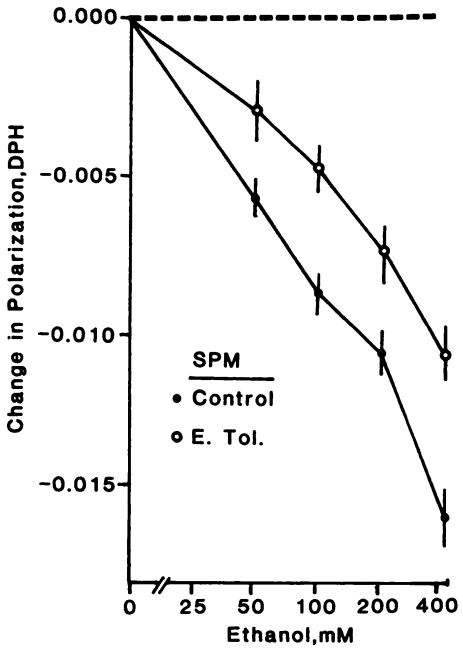

FIG. 3. *Effects of in vitro exposure to ethanol on the fluorescence polarization of DPH in synaptic membranes from controls* (●) *and ethanol tolerant-dependent* (○) *mice*

Assay temperature was 35°. Values represent means ± standard error of the mean for 12 different membrane preparations.

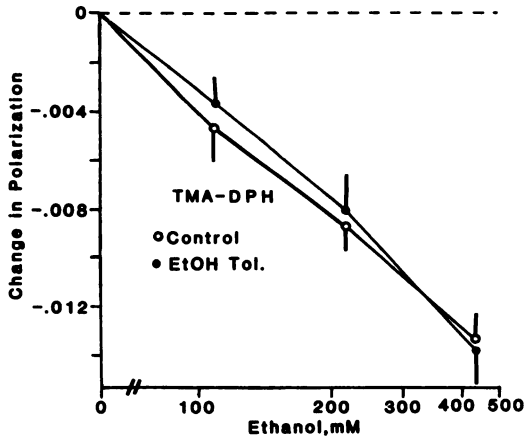

FIG. 4. *Effects of in vitro exposure to ethanol on the fluorescence polarization of TMA-DPH in synaptic membranes from control* (O) *and ethanol tolerant-dependent* (●) *mice*

Assay temperature was 35°. Values represent means ± standard error of the mean for six different membrane preparations.

creased membrane rigidity and membrane resistance to ethanol produced by chronic ethanol treatment was maintained for at least 8 hr after withdrawal of ethanol.

Effects of chronic ethanol consumption on the lipid composition of SPM. Synaptic membranes from control and ethanol tolerant-dependent mice did not differ in their content of lipid phosphate or gangliosides (Table 2) or in the distribution of lipid phosphate among the types of phospholipids (Table 3). Membrane cholesterol was reduced 19% by chronic alcohol treatment (Table 2). The acyl composition of each type of phospholipid was analyzed, and chronic ethanol was found to reduce the 22:6 and increase the 16:0 acyl groups of phosphatidylserine (Table 4). As a result, the ratio of long acyl groups (C-20 and C-22) to short acyl groups (C-16 and

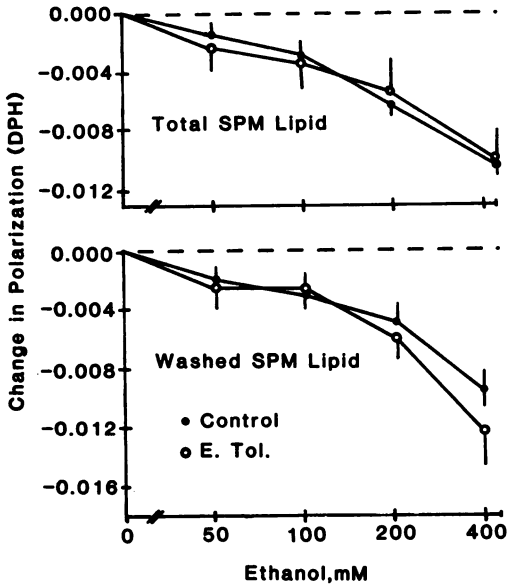

FIG. 5. *Effects of in vitro exposure to ethanol on the fluorescence polarization of DPH in lipids from control* (●) *or ethanol tolerant-dependent* (O) *mice*

Vesicles were formed from a total lipid extract (upper panel) or a washed lipid extract (lower panel) of the same membrane preparations reported in Fig. 3. Assay temperature was 35°.

TABLE 2

Effect of chronic ethanol treatment on the lipid composition of synaptic membranes

Brain SPM were obtained from DBA/2 mice fed an ethanol-containing diet or an isocaloric control diet for 7 days.

Parameter	Control[a]	Ethanol[a]
Lipid phosphate (μmoles/mg protein)	0.86 ± 0.11	0.94 ± 0.12
Cholesterol/phospholipid (molar ratio)	0.72 ± 0.03	0.58 ± 0.05[b]
Gangliosides (nmoles NANA[c]/mg protein)	106 ± 17	111 ± 14
Gangliosides (moles NANA/mole phospholipid)	0.12 ± 0.01	0.13 ± 0.01

[a] Values are means ± standard error of the mean, $n = 6$.
[b] Significantly different from control ($p < 0.05$).
[c] NANA, *N*-acetylneuraminic acid.

C-18) decreased from 0.64 (control) to 0.40 (ethanol), the ratio of unsaturated to saturated acyl groups decreased from 1.38 (control) to 1.08 (ethanol), and the double bond index (1) decreased from 232 (control) to 176 (ethanol). The acyl composition of phosphatidylcholine, phosphatidylethanolamine, and ethanolamine plasmalogen was not altered by chronic ethanol treatment (Table 4). The acyl composition of gangliosides was not affected by chronic ethanol administration. The major acyl groups of sphingomyelin (18:0 and 24:0) were not altered, but the content of 16:0, a minor constituent, was increased by chronic ethanol treatment (Table 5). Although ethanol treatment did not alter the sialic acid content of the SPM or the acyl composition of the gangliosides, it was important to determine whether the ganglioside composition was changed by ethanol exposure. Two-dimensional thin layer chromatography allowed separation and quantitation of 10 groups of gangliosides. The membrane content of these individual gangliosides was not affected by ethanol treatment (Table 6).

DISCUSSION

Administration of a liquid diet containing ethanol to mice resulted in the development of tolerance and dependence, in agreement with other reports (30–32). The ethanol ED_{50} for loss of righting reflex was chosen as a measure of alcohol sensitivity, as it can be determined within 3 min after i.p. injection of ethanol. In contrast to the more commonly used measure of "sleep time," the ED_{50} minimizes the possible influence of acute tolerance development and ethanol metabolism on the end-point. Chronic ethanol treatment produced a much larger change in the ethanol ED_{50} than on brain ethanol levels at regaining righting reflex. This difference may reflect the steep ethanol dose-response curve, which allows small changes in brain ethanol levels to produce large changes in behavioral parameters. This regimen of chronic alcohol administration does not alter ethanol metabolism[8] (30), and it is likely that the change in ethanol ED_{50} is an accurate reflection of cellular tolerance.

[8] R. A. Harris and M. A. Mitchell, unpublished results.

TABLE 3

Effect of chronic ethanol treatment on synaptic membrane phospholipid composition

Treatment	Phospholipid component[a]					
	PC	PS	EP	PE	SP	Other[b]
	% composition					
Ethanol	41.1 ± 0.8	6.9 ± 0.5	24.6 ± 2.2	20.7 ± 0.7	5.6 ± 0.2	4.3 ± 1.4
Control	39.0 ± 2.0	8.1 ± 0.7	24.4 ± 0.7	20.3 ± 0.8	5.2 ± 0.3	3.7 ± 1.5

[a] PC, Phosphatidylcholine; PS, phosphatidylserine; EP, ethanolamine plasmalogen; PE, phosphatidylethanolamine; SP, sphingomyelin. Values are means ± standard error of the mean, $n = 6$.

[b] Includes phosphatidic acid, phosphatidylinositol, and polyphosphatidylinositols.

TABLE 4

Effects of chronic ethanol treatment on the acyl composition of synaptic membrane phospholipids

Brain SPM were obtained from DBA/2 mice fed an ethanol-containing diet or an isocaloric control diet for 7 days.

Acyl group	Phosphatidylserine[a]		Phosphatidylcholine[a]		Phosphatidylethanolamine[a]		Ethanolamine plasmologen[a]	
	Control	Ethanol	Control	Ethanol	Control	Ethanol	Control	Ethanol
	% composition							
16:0	3.2 ± 0.9	11.6 ± 3.6[b]	43.1 ± 2.2	44.6 ± 0.4	6.5 ± 0.8	7.7 ± 0.4	1.2 ± 0.3	2.7 ± 0.4
18:0	38.7 ± 0.9	35.8 ± 3.6	14.6 ± 0.6	14.0 ± 0.2	33.3 ± 0.8	32.6 ± 0.5	2.2 ± 0.4	2.7 ± 0.6
18:1	17.7 ± 1.4	22.2 ± 2.1	31.8 ± 1.4	30.2 ± 0.6	14.7 ± 0.5	14.1 ± 0.5	22.3 ± 0.9	21.9 ± 1.2
18:2	ND[c]	ND	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
18:3(ω-3+ω-6)	1.5 ± 0.2	1.1 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.3 ± 0.2	1.2 ± 0.1	0.5 ± 1.0	8.5 ± 0.6
20:3(ω-6)	1.2 ± 0.2	1.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
20:4(ω-6)	5.0 ± 0.6	4.9 ± 0.4	3.8 ± 0.1	3.8 ± 0.1	12.1 ± 0.2	11.9 ± 0.2	15.0 ± 0.2	14.7 ± 0.2
20:5(ω-3+ω-6)	ND	ND	0.4 ± 0.1	0.4 ± 0.1	>0.5	>0.5	0.9 ± 0.1	0.6 ± 0.1
22:4(ω-6)	4.7 ± 0.6	3.8 ± 0.4	0.4 ± 0.1	0.4 ± 0.1	4.1 ± 0.4	4.5 ± 0.6	14.2 ± 0.5	13.3 ± 0.3
22:5(ω-3)	ND	ND	ND	ND	ND	ND	1.0 ± 0.1	0.9 ± 0.1
22:6(ω-3)	27.9 ± 1.8	18.8 ± 2.3[d]	3.3 ± 0.2	3.2 ± 0.1	26.9 ± 0.6	26.9 ± 1.0	34.3 ± 2.2	34.0 ± 2.9

[a] Values are means ± standard error of the mean, $n = 6$.

[b] Significantly different from control ($p < 0.05$).

[c] Not detectable.

[d] Significantly different from control ($p < 0.01$).

TABLE 5

Effects of chronic ethanol treatment on the acyl composition of synaptic membrane sphingolipids

Brain SPM were obtained from DBA/2 mice fed an ethanol-containing diet or an isocaloric control diet for 7 days.

Acyl group	Gangliosides[a]		Sphingomyelin[a]	
	Control	Ethanol	Control	Ethanol
	% composition			
16:0	8.9 ± 1.2	10.0 ± 2.1	0.8 ± 0.3	8.1 ± 3.6[b]
18:0	41.7 ± 2.6	41.6 ± 3.3	81.3 ± 4.6	75.3 ± 4.7
18:1	17.6 ± 1.6	16.8 ± 1.3	ND[c]	ND
20:0	0.8 ± 0.1	0.8 ± 0.1	<0.5	0.6 ± 0.2
22:0	1.3 ± 0.2	1.1 ± 0.1	2.1 ± 0.1	2.4 ± 0.1
22:1	8.6 ± 0.6	8.7 ± 0.5	ND	ND
24:0	1.3 ± 0.5	2.3 ± 0.6	2.9 ± 0.6	1.8 ± 0.2
24:1	5.5 ± 1.1	5.6 ± 1.3	15.1 ± 4.2	11.5 ± 1.7
26:0	2.8 ± 0.2	3.2 ± 0.9	ND	ND
26:1	14.0 ± 3.4	12.0 ± 2.4	ND	ND

[a] Values are means ± standard error of the mean, $n = 6$.

[b] Significantly different from control ($p < 0.05$).

[c] Not detectable.

TABLE 6

Effect of chronic ethanol treatment on the ganglioside composition of synaptic membranes

Brain SPM were obtained from DBA/2 mice fed an ethanol-containing diet or an isocaloric control diet for 7 days.

Ganglioside	Control[a]	Ethanol[a]
	% composition	
G_{Q1}	2.2 ± 0.3	1.7 ± 0.4
G_{T1b}	20.7 ± 0.6	24.7 ± 2.6
G_{D1b}	9.9 ± 0.6	11.4 ± 0.6
$G_{D2} + G_{T1a}$	2.2 ± 0.3	2.5 ± 0.3
G_{D1a}	27.3 ± 1.4	27.0 ± 1.6
G_3	3.0 ± 0.4	2.9 ± 0.5
G_{M1}	20.2 ± 0.4	20.3 ± 0.5
G_{M2}	2.7 ± 0.9	21. ± 0.5
$G_{M3} + G_{M4}$	9.5 ± 2.1	10.3 ± 2.0
Unknown	2.5 ± 0.7	2.4 ± 0.4

[a] Values are means ± standard errors of the mean, $n = 6$.

Synaptic membranes from brains of ethanol tolerant-dependent mice were more rigid than those of pair-fed controls. This is the opposite of the fluidizing effect produced by acute exposure to ethanol. The increased rigidity is about the same magnitude as the fluidization produced by 50 mM ethanol (4, 5), which is approximately the brain ethanol concentration produced by ingestion of the liquid diet (30). This indicates that the adaptive response is of the proper magnitude to offset the alcohol which reaches the brain. In addition, low concentrations of alcohols, barbiturates, phenytoin, or valproic acid can produce membrane fluidization that is sufficient to return the membrane order to control levels[8] (5). Each of

these membrane perturbants suppresses the alcohol withdrawal syndrome (37, 38), consistent with the hypothesis that signs of alcohol withrawal are a consequence of increased membrane rigidity. In addition, alcohol-dependent mice placed in a cold environment (which lowers body temperature and should increase membrane rigidity) displayed more severe signs of withdrawal than mice in a warm environment (30). The increased rigidity was detected by fluorescent probes (*cis*-parinarate, *trans*-parinarate, DPH) of the lower portions of the lipid acyl chains and by a probe (TMA-DPH) of the glycerol backbone and upper regions of the acyl chains (34–36). It is significant that similar changes were obtained with the DPH probes and the parinarates. Thus, the results cannot be attributed to the insertion of bulky phenyl groups into the membrane. These observations are also in agreement with the report of Lyon and Goldstein (3) that chronic ethanol treatment increases the order parameter of 12-doxylstearate, an EPR probe of the membrane core. Technical problems prevent the study of 12-doxylstearate at physiological temperatures (the experiments of Lyon and Goldstein were carried out at 19°), but the use of fluorescent probes allowed us to determine the temperature dependence of membrane viscosity. SPM from ethanol tolerant-dependent mice were more rigid than controls at all temperatures studied (15°–36°). Chronic ethanol ingestion has been reported to increase the order parameter of 5-doxylstearate, a probe of the acyl regions near the membrane surface, in some studies (6, 7) but not in others (2, 3).

In addition to altering the membrane rigidity, chronic ethanol ingestion also reduced the sensitivity of SPM to the disordering effects of ethanol. This attenuation of ethanol action was observed with DPH, but not TMA-DPH, suggesting that a different adaptation to ethanol occurred at the membrane core than at the surface. This conclusion is somewhat different from that reached by others using EPR probes who found that chronic ethanol treatment attenuated the ethanol-induced decrease in order parameter of 5-doxylstearate but not that of 12-doxylstearate (3). The differences between the results obtained with fluorescent and EPR probes may reflect non-equivalent membrane locations of the probes.

Vesicles formed from lipids extracted from SPM of control and ethanol tolerant-dependent mice did not differ in baseline rigidity or in their sensitivity to the fluidizing effects of ethanol. These results are not in agreement with those of Johnson *et al.* (9–11), who reported that chronic alcohol treatment increased lipid rigidity and ethanol resistance. However, the studies of Johnson *et al.* differ from ours in that they administered ethanol by repeated injection and selected only those animals that displayed a certain degree of tolerance. In addition, they used a crude mitochondrial preparation (P_2 fraction containing myelin, mitochondria, and synaptosomes) as their source of membrane lipids. Furthermore, the difference between control and chronic ethanol treatments reported by Johnson *et al.* (9, 10) for DPH polarization are only 0.001–0.002. The changes we observed in intact SPM were about 4-fold larger, and it is unlikely that we could detect changes of the magnitude reported by Johnson *et al.*

Although the physical properties of lipids extracted from SPM of control and chronic ethanol mice were not different, the changes in the physical properties of the intact SPM could still be due to changes in lipid composition. This would require that the lipids be properly oriented or arranged (e.g., vertical or lateral asymmetry, surrounding proteins, etc.) for the changes in lipid composition to be expressed as detectable changes in physical properties. To test this hypothesis, we examined the phospholipid, sphingolipid, and sterol composition of SPM from control and ethanol tolerant-dependent mice. Chronic ethanol ingestion resulted in a change in acyl composition that was selective for phosphatidylserine. Because phosphatidylserine comprises only about 8% of the SPM phospholipid, these changes would probably not be detectable in measurements of total acyl composition of SPM. This is in agreement with the negative results of Smith and Gerhart (13) and Wing *et al.* (17). In contrast, Littleton and John (14) found that ethanol exposure alters the acyl groups of synaptosomal phospholipids. Also, Sun and Sun (16) and Alling *et al.* (15) reported that chronic ethanol treatment changes the acyl composition of synaptosomal phosphatidylcholine and phosphatidylethanolamine. These changes are, however, small and inconsistent. For example, Sun and Sun (16) found that chronic ethanol ingestion *decreases* the 18:1 groups of phosphatidylcholine from 28.1% (control) to 25.9% (ethanol), whereas Alling *et al.* (15) reported that alcohol treatment *increases* this acyl group from 27.2% (control) to 28.3% (ethanol). These studies, together with the present data, indicate that chronic exposure to ethanol does not markedly alter the acyl composition of most SPM phospholipids but does produce selective changes in phosphatidylserine. We should note that none of these studies would be capable of detecting subtle changes in molecular species of phospholipids resulting from differences in the placement of the acyl groups on positions 1 and 2 of the glycerol moiety of a given phospholipid. Such changes in molecular species accompany temperature adaptation in *Tetrahymena pyriformis*, suggesting a role in homeoviscous adaptation (39).

Cholesterol content is an important determinant of membrane physical properties, and chronic ethanol treatment has been shown to increase synaptosomal cholesterol (12, 13). However, it is unlikely that decreased cholesterol was responsible for the changes in membrane physical properties in this study. Furthermore, others (3, 9) have demonstrated altered membrane rigidity and ethanol responsiveness in the absence of changes in cholesterol content.

In view of the limited effects of ethanol ingestion on phospholipids and cholesterol, we turned our attention to the remaining class of synaptic lipids, gangliosides. Gangliosides comprise the major sphingolipid in SPM and primarily located in the external half of the membrane bilayer, the phosphatidylcholine-rich region (21, 40). Studies with phosphatidylcholine liposomes indicate that gangliosides, particularly monosialogangliosides, in-

crease membrane rigidity and enhance the membrane-fluidizing effect of ethanol (19). The data suggest that changes in SPM ganglioside content and/or composition could play a role in tolerance/dependence development. However, we found no change in the lipid sialic acid content, the ganglioside composition, or the ganglioside acyl composition following chronic ethanol ingestion. In contrast, others have found changes in the total SPM sialic acid content (41). Our results suggest that these changes probably reside in the glycoproteins rather than the glycolipids.

The results of the present study, obtained with several fluorescent probes, demonstrate that chronic ethanol treatment increases the rigidity of synaptic membranes and decreases their sensitivity to the fluidizing action of ethanol. These findings are, for the most part, in agreement with results obtained with EPR probes. Despite these reliable changes in membrane physical properties, the membrane lipid composition is remarkably similar for SPM from control and ethanol tolerant-dependent mice. Changes in the acyl composition of phosphatidylserine may be partially responsible for the altered physical properties, as an increase in the proportion of saturated fatty acids would increase membrane rigidity, particularly in the membrane core. However, there must be additional factors, since the alterations found in intact membranes are not preserved in the extracted lipids. It is possible that chronic ethanol ingestion affects the properties of the membranes by altering the lipid arrangement (domains, asymmetry) or by altering the protein components of the membrane, which in turn influence the lipid order.

ACKNOWLEDGMENTS

We thank Gary Sieckmann and Pat Bruno for excellent technical assistance, and Genie Eckenfels for typing the manuscript.

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