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The Effect of 1-Propanol on the Rotational Mobility of *n*-(9-Anthroyloxy) stearic acid in Outer Monolayers of Neuronal and Model Membranes

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The aim of this study was to provide a basis for the molecular mechanism underlying the pharmacological action of ethanol. We studied the effects of 1-propanol on the location of *n*-(9-anthroyloxy)palmitic acid or stearic acid (*n*-AS) within the phospholipids of synaptosomal plasma membrane vesicles (SPMV). The SPMV were isolated from the bovine cerebral cortex and liposomes of total lipids (SPMVTL) and phospholipids (SPMVPL). 1-Propanol increased the rotational mobility of inner hydrocarbons, while decreasing the mobility of membrane interface, in native and model membranes. The degree of rotational mobility varied with the number of carbon atoms at positions 16, 12, 9, 6 and 2 in the aliphatic chain of phospholipids in the neuronal and model membranes. The sensitivity of increasing or decreasing rotational mobility of hydrocarbon interior or surface by 1-propanol varied with the neuronal and model membranes in the following order: SPMV, SPMVPL and SPMVTL.

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Introduction

There are several hypotheses about the molecular mechanism of action of ethanol, including lipid and protein theory. However, the precise location of molecular action has been controversial to date. The current consensus is that ethanol probably has a site of action that is located within the cell membrane at the synapses. The debate continued as to whether the site was exclusively located in the lipid bilayer, the hydrophobic region of the protein, or the membrane protein-lipid interface.

In most studies, the effect of ethanol on the fluidity of the membrane was not the rotational mobility of the individual acyl chains of the outer layer, but the rotational and lateral mobility of the bulk or individual monolayer. Studies on the structure-activity relationship of n-alkanol are expected to be a very important material for studying the mechanism of ethanol action.

Previous studies have shown that the fluorophores of anthrolyoxy derivatives are in a series of graded positions from the surface to the center of the lipid bilayer structure (or a series of anthroyloxy fatty acids indicate that the depth of the group is almost linearly related to the number of carbon atoms with

carboxy group) [1-4]. Fluorophores of the anthroyloxy group can be used to distinguish whether the bilayer has a fluidity gradient across it because the anthroyloxy group can be located at different positions in the stearic acid moiety [3-6]. These probes have been proposed primarily to measure the dynamic component of membrane fluidity [5].

The aim of this research is to provide a basis for studying the molecular mechanism of pharmacological action of ethanol. In this study, 1-propanol was investigated through the study of the rotational motility of the hydrocarbons inside and at the pole (membrane interface, surface area) of natural and model membranes with different flow properties. Sensitivity between intrinsic membranes for fluidizing or ordering effect of 1-propanol. 16-(9-anthroyloxy)palmitic acid (16-AP), 12-(9-anthroyloxy)stearic acid (12-AS), 9-(9-anthroyloxy)stearic acid (9-AS), 6-(9-anthroyloxy)stearic acid (6-AS) and 2-(9-anthroyloxy)stearic acid (2-AS) reflecting the rotational mobility at positions 16, 12, 9, 6 and 2 of the existing aliphatic chain present in phospholipids of synaptosomal plasma membrane vesicles (SPMVs) isolated from bovine cerebral cortex and liposomes of total lipids (SPMVTL) and phospholipids (SPMVPL) extracted from SPMVs.

Materials and Methods

Materials

The fluorescent anthroyloxy palmitate or stearate probes, 16-AP, 12-AS, 9-AS, 6-AS and 2-AS were obtained from Molecular Probes (Eugene, OR). 1-Propanol was purchased from Fluka (Buchs,Switzerland). Other reagents were obtained from Sigma (St.Louis, MO) and were analytical grade.

SPMV preparation

The SPMV was prepared according to the procedures reported in earlier studies [7,8]. The specific activities of Na,K-ATPase, acetylcholinesterase and 5'-nucleotidase in the plasma membrane fraction were approximately 4-, 2.5- and 3-times higher than those of crude homoginitic acid. The electron microscopic examination of SPMV showed very high purity. The vesicles isolated by size showed a homogeneous distribution and no longer showed the presence of intracellular organelles or leakage. The protein concentration was measured by Lowry *et al.* [9] using bovine serum albumin (BSA) as a standard.

Liposome preparation

Total lipids were extracted from the SPMV as previously described [7]. The cholesterol content of extracted total lipids was determined by the Liebermann-Buchard reaction [10]. The phospholipid was quantified by measuring the amount of inorganic phosphate [11] after hydrolysis of the phospholipids at 180°C in 70% $HClO_4$ [12].

The properties of the lipid samples, such as size, lamellarity, radius of curvature, and shape are strongly dependent on the method used to form the vesicles [6,13,14]. As a result of the above process, the parameters that characterize the lipid phase equilibrium in lipid mixtures are affected by the lipid sample characteristics. Because the size of GUVs is in the same order as the cell size, GUVs have been intensively investigated in a variety of areas that focus on membrane behavior [6,14,15]. Stock solutions of total lipids or phospholipids were made in chloroform. The concentration of the lipid stock solutions was 0.2 mg/ml. Giant unilamellar vesicles (GUVs: SPMVTL or SPMVPL) with a mean diameter of 45 mm were prepared by the developed method[16-18]. The properties of size specimens, such as radius of curvature and shape, are used to determine the usage [6,13,14]. As a result of the manufacturing process, the parameters that characterize the lipid phase equilibrium in the lipid mixture are affected by the lipid sample characteristics. To grow the GUV, the previously described [19,20] special temperature control chamber was used. The experiments was performed in the same chamber after vesicle formation using an inverted microscope (Axiovert35: Zeiss, Thornwood, NY). The following steps were used to prepare the GUVs. 1) ~ 3 μl of lipid storage solution was spread on each Pt wire with an N2 stream. To remove residues of organic solvents, we put ~ 2h of chamberinal ionizer. 2) Seal the bottom of the chamber with a coverslip to add an aqueous solvent inside the chamber (Milliporewater 17.5MW/cm). Millipore water was previously heated to the desired temperature (80°C for SPMVTL and 60°C for SPMVPL) and then added enough water to cover the Pt wires. Immediately after this step, the Pt wire was connected to a function generator (Hewlett-Packard, Santa Clara, CA), and a low-frequency AC field (sinusoidal wave function with a frequency of 10 Hz and an amplitude of 3 V) was applied for 90 min. After the vesicle formation, the AC field was turned off.

Fluorescence measurements

The fluorescence measurements were performed using

modified methods of previous [5,6,21]. The SPMV were suspended in phosphate-buffersaline (PBS) to concentration 50μg of protein/ml. The liposomes (SPMVTL and SPMVPL) were suspended in in PBS to give a total lipid or total phospholipid concentration of 0.2 mg. Stock solutions of the in Methanol (2×10^{-5}M) of 16-AP, 12-AS, 9-AS, 6-AS and 2-AS was prepared and stored in a cold and dark place. Aliquots were added to the solutions of the native membrane to give a final concentrations of the 16-AP, 12-AS, 9-AS, 6-AS and 2-AS became 4×10^{-8}M (for SPMV) or 2×10^{-8}M (for SPMVTL and SPMVPL) incorporated the probes. The mixture was stirred at room temperature for 20 min to reduce the methanol concentration that might alter the rotational mobility of the SPMV, SPMVTL and SPMVPL. The mixture was also bubbled with dry nitrogen for 1 min at 20 min intervals to eliminate oxygen which might act as a quencher. To ensure complete removal of 1-propanol residue in the mixture, the prepared mixtures were subjected to exhausted stirring for more than 2 hr which have shown the same results as the mixtures stirred for 20 min. A concentrated solution of 1-propanol was made in PBS and added to the labeled membrane suspension to provide the desired concentration of anesthetic. The pH of the buffered sample was not significantly changed by addition of 1-propanol.

Fluorescence measurements were carried out with a Multi Frequency Cross-Correlation Phase and Modulation Fluorometer (ISS K2-003), equipped with a thermostated cell holder and performed at pH 7.4 (37 ± 0.1°C). The fluorescent probes, 16-AP, 12-AS, 9-AS, 6-AS and 2-AS, were excited at 360 nm (4 nm slit width) and those emissions recorded at 445 nm (8 nm slit width) through a sharp cut-off filter (Schott KV418). Corrections for light scattering (membrane suspensions without fluorescent probes) and the surrounding medium (quantified by pelleting the membranes after each estimation) were routinely performed, and the combined corrections was observed for anthroyloxy palmitate or stearate-loaded suspensions less than 9% of the total fluorescence intensity. The intensity of the components of the fluorescence which were parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the direction of the vertically polarized excitation light, was determined by measuring the light emitted through vertically and horizontally oriented polarizers. Polarization (P) was obtained from intensity measurements using $P = (I_{\parallel} - GI_{\perp})/(I_{\parallel} + GI_{\perp})$, where G is a grating correction factor for the transmission efficiency of the monochromator's for vertical and horizontal polarized light. This value is given by the fluorescence intensity ratio of the vertical and horizontal components when the exciting light is polarized in the horizontal direction. The polarization was expressed as anisotropy [$r = 2P/(3-P)$] of 16-AP, 12-AS, 9-AS, 6-AS and 2-AS.

Results

In this study, we investigated the amphiphilic effects of cation 1-propanol on the differential rotational mobility between interface and hydrocarbon interior of SPMV, SPMVTL and SPMVPL using fluorescence probe technique. In order to measure the effect of the 1-propanol on above-described rotational motility, it must first be verified that the drug does not directly interact with the fluorescent probe to quench the fluorescence. Compensation of absorbance by the drug quenching of the fluorescence intensity is not observed for all concentrations of 1-propanol tested. In addition, if direct quenching of 16-AP, 12-AS, 9-AS, 6-AS and 2-AS by the 1-propanol occurred, fluorescence lifetime would decrease. However, the fluorescence lifetime of 16-AP was not changed by the drug in the SPMV. Direct quenching of probe fluorescence by the drug used in the current experiments was excluded.

The anisotropy (r) values of 16-AP for hydrocarbon interior of intact SPMV, SPMVTL and SPMVPL were 0.092 ± 0.002 (n = 5), 0.070 ± 0.001 (n = 5) and 0.047 ± 0.001 (n = 5) at 37°C (pH 7.4) respectively (Table 1). In contrast, the values

Table 1. Fluorescence parameters of 16-AP, 12-AS, 9-AS, 6-AS and 2-AS in SPMV and SPMVTL and SPMVPL

Membranes	Parameter	16-AP	12-AS	9-AS	6-AS	2-AS
SPMV	Anisotropy	0.092 ± 0.002	0.099 ± 0.002	0.108 ± 0.001	0.115 ± 0.002	0.126 ± 0.002
SPMVTL	Anisotropy	0.070 ± 0.002	0.078 ± 0.001	0.098 ± 0.001	0.104 ± 0.003	0.114 ± 0.001
SPMVPL	Anisotropy	0.047 ± 0.001	0.051 ± 0.001	0.082 ± 0.001	0.087 ± 0.001	0.096 ± 0.001

Fluorescence measurements were performed at 37°C (pH 7.4). Values represent the mean ± SEM of 5 sample determinations.

of 2-AS for interface of intact SPMV, SPMVTL and SPMVPL were 0.126 ± 0.002 (n = 5), 0.114 ± 0.001 (n = 5), 0.096 ± 0.001 (n = 5) at 37°C (pH 7.4) respectively (Table 1). This means that rotational mobility of hydrocarbon interior is faster than that of membrane interface.

The 1-propanol may induce disordering or ordering of host lipids. Ordering occurs at membrane interface, whereas disordering occurs deep within the acyl chains. 1-Propanol has a large disordering effects on hydrocarbon interior of native and model membranes, but the ordering effects of 1-propanol on membrane interface are minor (pH 7.4, 37°C).

Ordering effects of 1−propanol on the rotational mobility of the membrane interface

The effect of the 1-propanol is shown the effect of the 2-AS anisotropy (*r*) at the interface SPMV, SPMVTL and SPMVPL Figure 1-3. The 1-propanol increased the anisotropy (*r*) of the 2-AS (decreased rotational mobility) in interface of SPMV, SPMVTL and SPMVPL in a concentration-dependent manner. A significant increase in the anisotropy (*r*) value by 1-propanol was observed even at 10 mM (Figs. 1-3), respectively. The anisotropy (*r*) values of the 2-AS in interface of SPMV,

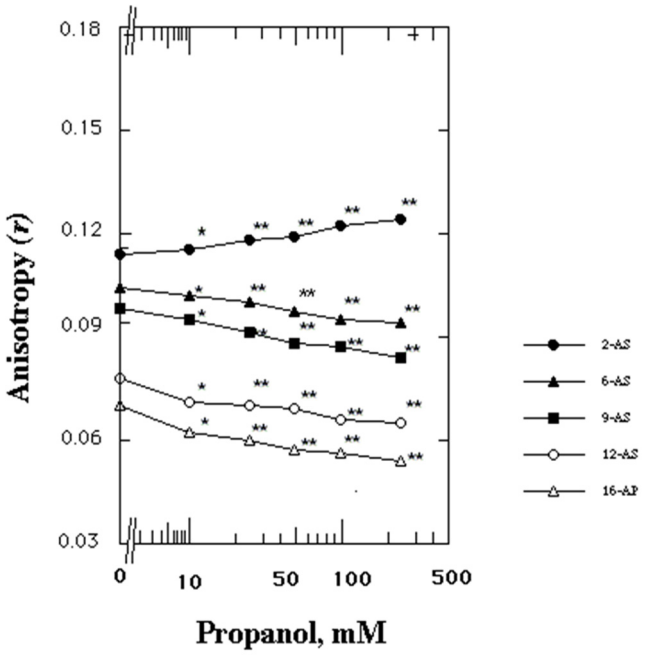

Fig. 2. The effect of 1-propanol on the anisotropy (*r*) of the 2-AS, 6-AS, 9-AS, 12-AS and 16-AP in the SPMVTL. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 sample determinations. An asterisk and double asterisks signify $P< 0.05$ and $P < 0.01$, respectively, compared to control according to Student's *t*-test.

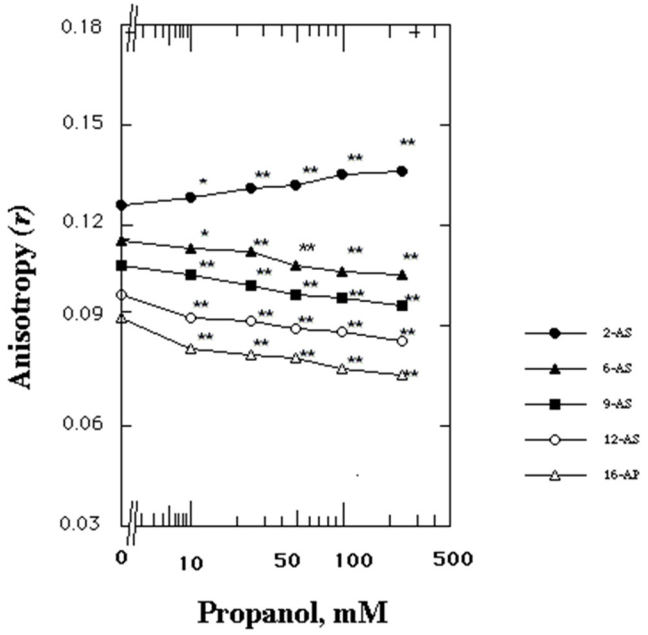

Fig. 1. The effect of 1-propanol on the anisotropy (*r*) of the 2-AS, 6-AS, 9-AS, 12-AS and 16-AP in the SPMV. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 sample determinations. An asterisk and double asterisks signify $P< 0.05$ and $P < 0.01$, respectively, compared to control according to Student's *t*-test.

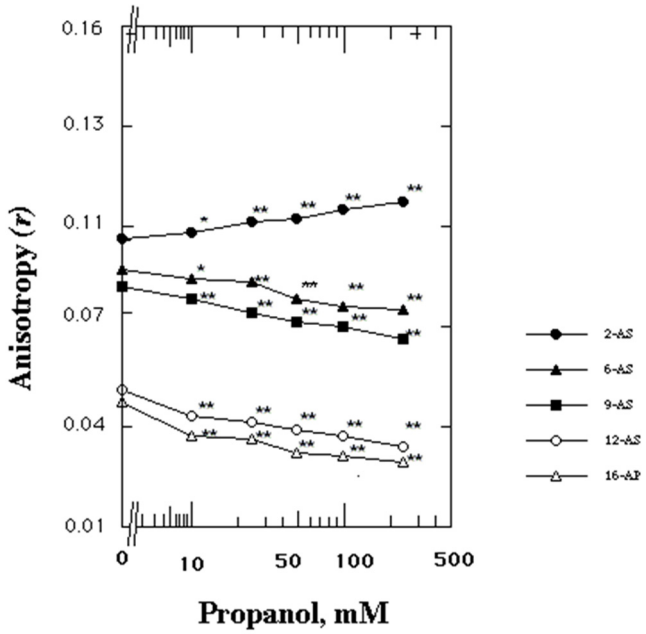

Fig. 3. The effect of 1-propanol on the anisotropy (*r*) of the 2-AS, 6-AS, 9-AS, 12-AS and 16-AP in the SPMVPL. Fluorescence measurements were performed at 37°C (pH 7.4). Each point represents the mean ± SEM of 5 sample determinations. An asterisk and double asterisks signify $P< 0.05$ and $P < 0.01$, respectively, compared to control according to Student's *t*-test.

SPMVTL and SPMVPL were raise by 0.006, 0.005 and 0.006, respectively, than those in the same region when 50mM 1-propanol was added. Variations in the anisotropy (*r*) values were also noticed by the aforementioned temperature change. At 37°C (pH 7.4), the anisotropy (*r*) of the 2-AS in interface of SPMV, SPMVTL and SPMVPL are 0.126 ± 0.002 (n = 5), 0.114 ± 0.001 (n = 5), 0.096 ± 0.001 (n = 5). On the other hand, at 25°C (pH 7.4), the anisotropy (*r*) of the 2-AS in interface of SPMV, SPMVTL and SPMVPL are 0.165 ± 0.003 (n = 5), 0.157 ± 0.002, 0.142 ± 0.001. Based on the results obtained at different temperatures, the observed effects by the addition of 50 mM 1-propanol, 0.006, 0.005 and 0.006 were comparable to the effect of temperature changes as much as about 1.8, 1.4 and 1.7°C, respectively.

Disordering effects of 1−propanol on the rotational mobility of the hydrocarbon interior

Figs. 1-3 show the effect of increasing concentrations of the 1-propanol on the anisotropy (*r*) of the 16-AP, 12-AS, 9-AS and 6-AS in the hydrocarbon interior of SPMV, SPMVTL and SPMVPL. The 1-propanol decreased the anisotropy (*r*) of the 16-AP, 12-AS, 9-AS and 6-AS (increased rotational mobility) in a dose-dependent manner. The significant decreases in the anisotropy (*r*) values by the 1-propanol in the SPMV, SPMVTL and SPMVPL was observed at concentrations as low as 10 mM, respectively (Figs. 1-3). The magnitude of the increased rotational mobility by the 1-propanol was in the order at the 16, 12, 9 and 6 positions of aliphatic chains in the phospholipids of neuronal and model membranes.

The differences in the anisotropy (*r*) values of the 16-AP found in hydrocarbon interior of SPMV, SPMVTL and SPMVPL before and after adding 50 mM 1-propanol were 0.012, 0.013 and 0.015. These can be explained by comparing effects of temperature on this parameter. The anisotropy (*r*) of the 16-AP in hydrocarbon interior of SPMV, SPMVTL and SPMVPL are 0.092 ± 0.002 (n = 5), 0.070 ± 0.001 (n = 5), 0.047 ± 0.001 (n = 5) at 37°C (pH 7.4), respectively. The anisotropy (*r*) of the 16-AP in hydrocarbon interior of SPMV, SPMVTL and SPMVPL are 0.122 ± 0.003 (n = 5), 0.106 ± 0.001 (n = 5) and 0.086 ± 0.001 (n = 5) at 25°C (pH 7.4), respectively. Thus, the differences in the anisotropy (*r*) values at 16 positions in hydrocarbon interior of SPMV, SPMVTL and SPMVPL before and after addition of 50 mM 1-propanol was 0.012, 0.013 and 0.015, rewpectively, about 4.8, 4.3 and 4.6°C goes up.

Discussion

We have paid a special attention to studies by Villalaín and Prieto [1] in several studies on membrane 2-AS distribution [1-4,22,23]. The research [1] clearly showed the distribution region. As the chromophore is adsorbed to the membrane interface, 2-AS is reported to be a unique member of the probe group. Membrane interface is difficult to define precisely and contain carbonyl groups that are predominantly involved in hydrogen bonding or pole sites near phospholipid head groups. The degrees of rotational motility of the phospholipids constituting the neuronal and model membranes were found at 16, 12, 9, 6 and 2 positions of the aliphatic chains in the phospholipids, respectively. Using the membrane interface probe 2-AS, we found that 1-propanol decreased the rotational mobility of lipids in the interface of neuronal and model membranes. On the contrary, using the hydrophobic interior probe 16-AP, 12-AS, 9-AS and 6-AS, 1-propanol has been shown to increase the rotational mobility of lipids in the hydrophobic regions of neurons and model membranes. We have also found that the magnitude of the effect of 1-propanol is greater than that of the model membrane in terms of increasing or decreasing the mobility of neuronal and model membrane lipid bilayers by 1-propanol.

The 1-propanol used in this study dose-dependently lowered the anisotropy (*r*) values of 16-AP, 12-AS, 9-AS and 6-AS in hydrocarbon interior of SPMV, SPMVTL and SPMVPL but increased anisotropy (*r*) of 2-AS in interface of the native and model membranes.

Our data suggest that the observed anisotropy (*r*) values reflect differences in the rates of hydrocarbon interior and interface of SPMV, SPMVTL and SPMVPL. This is due to differences in the intrinsic component and/or the structure in interface and hydrocarbon interior of the native and model membranes. The mechanism of the action(s) of 1-propanol on disordering and ordering effects on the neuronal and model membranes is not well-known. Water combines with the head group region of phospholipids via hydrogen bonding[24]. 1-Propanol bind (the competitive binding of the 1-propanol and water) strongly to the phosphate moiety of the phospholipids in membrane interface and weakly to the carbonyl group in competition with water, and effectively establish formation of hydrogen bonds with the carbonyl moiety, which is associated with a significant change in hydration of the 1-propanol molecules themselves [25]. Incorporation of 1-propanol into the

native and model membranes cause alterations of the interface's charge density of the membrane, and a conformational change in phospholipid head groups [25]. At the same time, 1- propanol may exert a significant influence on hydration of the lipid bilayer. As a result, such competitive binding decreases rotational mobility and increases hydrophobicity [25]. The interaction between the two steps leads to the reconstruction of molecules between the molecules of molecules in relation to the free molecules of water molecules and the protein molecules associated with free molecules associated with the liberation of water molecules. Interaction also changes the orientation of the P-N dipole in the phospholipid molecules, causing confusion in the presence of hydrocarbons and affecting the transport of internal + and internal transport.

The sensitivities to the increasing effect of the rotational mobility of the hydrocarbon interior by the 1-propanol differed depending on the native and model membranes in the descending order of the SPMV, SPMVPL and SPMVTL. As the results of this study, there no doubt that the 1-propanol increases the rotational mobility of the hydrocarbon interior of the membranes. What could be the effect on neuronal membranes where phospholipids, cholesterol and proteins are co-present are greater compared to the effect on model membranes where the protein is not co-present? It is presumed because proteins expand the effect of 1-propanol on lipids through protein-lipid interaction. These effects are not solely due to the influence of the 1-propanol on lipids, but they are magnified by the interaction between lipids, proteins and water. Water plays a fundamental role in membrane structure in that it drives the formation of the lipid bilayer, with a polar surface facing the aqueous environment and a hydrophobic interior containing the fatty acyl chains and transmembrane proteins. Typically, the structure and dynamics of proteins are predominantly governed by interaction with water [27]. Water penetrates into the lipid layer of the lipid layer and penetrates deeper into the area of the lipid chain, even though the lipid layer is at least as deep as the glycerol backbone. The water in the protein-lipids is an additional factor affecting the geological layer structure. The introduction of small peptides, consisting of three amino acids, can cause a shift of water deeper into the bilayer, indicating increased hydration [28]. Altered hydration may have marked effects on membrane protein/lipid functioning, possibly due to the formation of hydrogen bonds between the interchain water and protein amino acid side chains facing/lipid acyl chains facing into the hydrophobic interior of the membrane. It is possible that the proteins organize the lipid in a way that makes them more susceptible to the drug.

Ethanol increased the lateral and the rotational mobilities of plasma membrane vesicles (CHOK1-PMV) of cultured Chinese hamster ovary K1 cells [29], the plasma membrane vesicles (ATCC-PMV) of cultured hybridoma cells (ATCCT-1B216) [30], plasma membrane vesicles of the cultured mouse myeloma cell line Sp2/0-Ag14 [31] and SPMV [32]. Ethanol had a greater effect on increasing the range of rotational mobility of the outer compared to the inner monolayer of CHOK1-PMV [29], ATCC-PMV [30], Sp2/0-Ag14 [31] and SPMV [32]. Furthermore, as mentioned in the introduction, a pathway for ethanol metabolism, where the product was anunusual phospholipid, phosphatidylethanol (PET) [33], has been reported. Judging from the results of the present study, as well as those from other studies [33-35], there is a good possibility that the effect of ethanol on CNS is not only direct action on neuronal membrane proteins, but also the action of neuronal membrane lipids.

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Conflict of interest

The authors declare that they have no conflicting interest.

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