

Organization and Dynamics of Hippocampal Membranes in a Depth-Dependent Manner: An Electron Spin Resonance Study

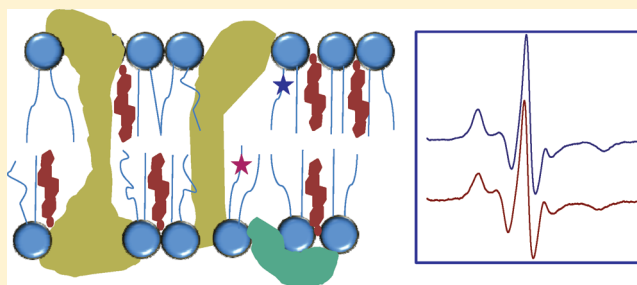
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S Supporting Information

ABSTRACT: Organization and dynamics of neuronal membranes represent crucial determinants for the function of neuronal receptors and signal transduction. Previous work from our laboratory has established hippocampal membranes as a convenient natural source for studying neuronal receptors. In this work, we have monitored the organization and dynamics of hippocampal membranes and their modulation by cholesterol and protein content utilizing location (depth)-specific spin-labeled phospholipids by ESR spectroscopy. The choice of ESR spectroscopy is appropriate due to slow diffusion encountered in crowded environments of neuronal membranes. Analysis of ESR spectra shows that cholesterol increases hippocampal membrane order while membrane proteins increase lipid dynamics resulting in disordered membranes. These results are relevant in understanding the complex organization and dynamics of hippocampal membranes. Our results are significant in the overall context of membrane organization under low cholesterol conditions and could have implications in neuronal diseases characterized by low cholesterol conditions due to defective cholesterol metabolism.



■ INTRODUCTION

Biological membranes are complex noncovalent assemblies of a diverse variety of lipids and proteins that allow cellular compartmentalization, thereby imparting an identity to the cell. The lipid composition of cells that makes up the nervous system is unique and has been correlated with increased complexity in the organization of the nervous system during evolution.¹ The nervous system characteristically contains a very high concentration of lipids and displays remarkable lipid diversity.² Cholesterol is an important lipid in this context since it is known to regulate the function of neuronal receptors,^{3–5} thereby affecting neurotransmission and giving rise to mood and anxiety disorders.⁶ Cholesterol is often found distributed nonrandomly in domains in biological and model membranes.^{7–9} Many of these domains (sometimes termed as 'lipid rafts'¹⁰) are thought to be important for the maintenance of membrane structure and function, although characterizing the spatiotemporal resolution of these domains has proven to be challenging.^{11–13} A unique property of cholesterol that contributes to its capacity to form membrane domains is its ability to form a liquid-ordered-like phase in higher eukaryotic plasma membranes.¹⁴ The idea of such specialized membrane domains assumes significance in cell biology since physiologically important functions such as membrane sorting and trafficking, signal transduction processes, and the entry of pathogens have been attributed to these domains.^{15,16} Interestingly, a number of neurological diseases share a common etiology of defective cholesterol metabolism in the

brain,¹⁷ yet the organization and dynamics of neuronal membranes as a consequence of alterations in membrane cholesterol is poorly understood.^{18–20}

Previous work from our laboratory has established native hippocampal membranes as a convenient natural source for exploring the interaction of the serotonin_{1A} receptor, an important member of the seven transmembrane domain G-protein coupled receptor family, with membrane lipids.^{21,22} Interestingly, we have demonstrated the requirement of membrane cholesterol in modulating the ligand binding function of the serotonin_{1A} receptor utilizing a variety of approaches.^{4,5,22–25} In order to correlate these cholesterol-dependent functional changes with alterations in membrane organization and dynamics, we have earlier utilized fluorescence-based approaches.^{26–29} However, fluorescence spectroscopic approaches can only provide information in a relatively fast (~nanoseconds) time scale. A comprehensive understanding of the organization and dynamics of biological membranes requires a wide range of spatiotemporal scales.^{12,13} In this work, we have therefore monitored the organization and dynamics of hippocampal membranes and their modulation by cholesterol and protein content, utilizing approaches based on electron spin resonance (ESR). ESR provides information in a relatively slow time scale and therefore would involve more

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averaging (compared to fluorescence spectroscopy), which could be crucial in a slow diffusing system such as natural membranes of neuronal origin.^{30,31} In addition, since the membrane is considered to be a two-dimensional anisotropic asymmetric fluid, any possible change in membrane organization and dynamics may not be uniform and restricted to a unique location in the membrane. For example, we have previously shown that stress such as heat shock can induce anisotropic changes in membrane organization, i.e., the change in membrane organization is different when monitored in different positions (depths) in adult rat liver cell plasma membranes.³² We therefore monitored the organization and dynamics of hippocampal membranes and their modulation by cholesterol and protein content utilizing depth-specific spin-labeled phospholipids, 5- and 14-PC (see Figure 1). The 5- and

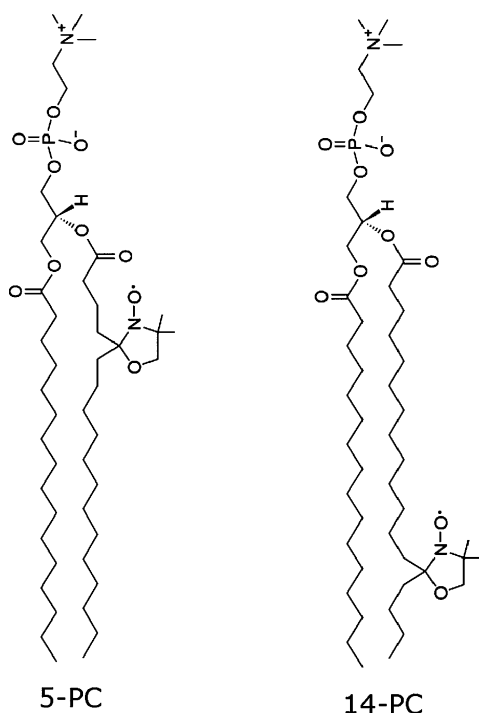


Figure 1. Chemical structure of spin labeled phospholipids.

14-PCs contain the stable doxyl nitroxide spin label on the 5th and 14th carbon atoms of the *sn*-2 acyl chain of the phospholipid, respectively. While 5-PC provides information on the order and dynamics at the membrane interface, 14-PC reports on the mobility and dynamics near the more isotropic center of the bilayer.^{33,34} The results obtained indicate that both cholesterol and proteins modulate hippocampal membrane dynamics. While cholesterol increases membrane order, membrane proteins appear to increase membrane lipid dynamics by disturbing the membrane order.

EXPERIMENTAL SECTION

Materials. Cholesterol, methyl- β -cyclodextrin (M β CD), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), EDTA, EGTA, iodoacetamide, phenylmethylsulfonyl fluoride (PMSF), sucrose, sodium azide, Na₂HPO₄, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). Bicinchoninic acid (BCA) reagent for protein estimation was from Pierce (Rockford, IL). Amplex Red cholesterol assay kit was from Molecular Probes (Eugene, OR). 1-Palmitoyl-2-(5-doxy)-

stearoyl-*sn*-glycero-3-phosphocholine (5-PC) and 1-palmitoyl-2-(14-doxy)stearoyl-*sn*-glycero-3-phosphocholine (14-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death, and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at -70°C until further use.

Preparation of Native Hippocampal Membranes.

Native hippocampal membranes were prepared from frozen hippocampal tissue as previously described.²⁶ Native membranes were suspended in a minimum volume of 50 mM Tris, pH 7.4, buffer containing 1 mM EDTA, homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen, and stored at -70°C . Protein concentration was assayed using the BCA reagent.³⁵

Cholesterol Depletion of Native Membranes.

Native hippocampal membranes were depleted of cholesterol using M β CD as described previously.^{22,36} Briefly, membranes with a total protein concentration of ~ 2 mg/mL were treated with different concentrations of M β CD in 50 mM Tris buffer (pH 7.4) at 25°C in a temperature controlled water bath with constant shaking for 1 h. Membranes were then spun down at 50 000g for 5 min, washed once with Tris buffer, and resuspended in the same buffer. Cholesterol was estimated using the Amplex Red cholesterol assay kit.³⁷

Lipid Extraction from Native and Cholesterol-Depleted Membranes. Lipid extraction was carried out according to the method of Bligh and Dyer³⁸ from native hippocampal membranes with some modifications. In order to yield efficient extraction of total lipids, hippocampal membranes were successively treated with varying ratios of methanol–chloroform (2:1, 1:1, and 1:2, v/v). Subsequently, water–chloroform (1:1, v/v) was added and organic and aqueous phases were separated upon centrifuging the samples at low speed. In order to retrieve total lipids, the lower layer of organic phase was isolated and dried off under nitrogen at $\sim 45^{\circ}\text{C}$. After further drying under a high vacuum for 6 h, the lipid extract was dissolved in a mixture of chloroform–methanol (1:1, v/v).

Estimation of Phospholipids. Concentration of lipid phosphate was determined subsequent to total digestion by perchloric acid,³⁹ using Na₂HPO₄ as standard. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings. The phospholipid content of native membranes is typically ~ 960 nmol/mg of total protein.³⁶

Sample Preparation. Spin-labeled lipids from a methanolic stock solution were added to hippocampal membranes containing ~ 2.5 mg total protein (~ 2.5 μmol phospholipids). The amount of spin label added was such that the final probe concentration was ~ 1 mol % with respect to the total phospholipid content. This ensures optimal ESR signal with negligible membrane perturbation. In case of lipid extracts, ~ 2.5 μmol of total phospholipids in chloroform–methanol (1:1, v/v) were mixed well with 25 nmol of the spin-labeled phospholipid (5-PC or 14-PC) and dried under a stream of nitrogen while being warmed gently ($\sim 45^{\circ}\text{C}$). The residual solvent was removed by drying under vacuum for 6 h. The lipid film obtained was then hydrated by adding 1 mL of 50 mM

Tris, pH 7.4, buffer containing 1 mM EDTA at $\sim 70^\circ\text{C}$ while being intermittently vortexed for 3 min to disperse the lipid and form homogeneous multilamellar vesicles (MLVs). MLVs were kept at $\sim 70^\circ\text{C}$ for an additional hour to ensure proper swelling as the vesicles were formed. Such high temperatures are necessary for hydrating the samples due to the presence of lipids with high melting temperature in neuronal tissues.⁴⁰ After vortexing, samples were transferred into a glass capillary (1 mm inside diameter (ID)), sealed at one end, and pelleted by centrifugation in a benchtop centrifuge. The excess supernatant was removed, and the capillary was sealed and stored at 4°C . Samples were always prepared on the day of the measurement and were never stored for more than 24 h before measurement.

ESR Spectroscopy. ESR spectra were recorded on a JEOL JES-FA 200 ESR spectrometer operating at 9 GHz. Samples in 1 mm ID glass capillaries, prepared as described above, were placed in a standard quartz ESR tube. The following instrumental settings were used for all measurements: scan width, 100 G; scan time, 4 min; number of accumulations, 4; time constant, 1 s; modulation width, 2 G; microwave power, 5 mW. Spectra were recorded at $25 \pm 0.2^\circ\text{C}$, and temperature was maintained constant during the measurement with a temperature-controller attached to the ESR spectrometer. Temperature was measured by a thermocouple placed close to the sample tube.

Nonlinear Least-Squares Analysis of ESR Spectra. ESR spectra of 5- and 14-PC dispersed in hippocampal membranes and liposomes of lipid extract were analyzed by a nonlinear least-squares (NLLS) method, based on the stochastic Liouville equation.^{41,42} Simulations were carried out using the latest version of the ESR fitting program⁴³ configured to run on a computer running on a Windows operating system. The program package (PC.NEW) is available from the Advanced Centre for ESR Technology (ACERT) at Cornell University (Ithaca, NY; http://www.acert.cornell.edu/index_files/acert_resources.php). In this analysis, values of the hyperfine tensors (A_{xx} , A_{yy} , A_{zz}) and g-tensor (g_{xx} , g_{yy} , g_{zz}) are kept fixed for each simulation. These parameters are slightly modified if fits obtained are not satisfactory, and the simulation is repeated until satisfactory fits are obtained. The optimized values are shown in Table S1 (Supporting Information) and were used for all spectra of a particular spin-labeled phospholipid. The values of the rotational diffusion coefficients and the terms that describe Gaussian inhomogeneous broadening and coefficients for orienting potential were varied.⁴³ The data points were reduced to 1024 by averaging the original data points from spectra. NLLS analysis of a spectrum provides the values of two important parameters that describe the dynamic order of the membrane lipids. These are (i) the rotational diffusion coefficient (R_\perp) of the nitroxide radical around the axis perpendicular to the mean symmetry axis for rotation (it represents the principal component of the rotational diffusion tensor of the nitroxide radical⁴⁴) and (ii) the order parameter (S_0), which is a measure of the angular extent of the rotational diffusion of the nitroxide moiety relative to the membrane normal.

RESULTS AND DISCUSSION

M β CD is a water-soluble compound and has previously been shown to selectively and efficiently extract cholesterol from hippocampal membranes by including it in a central nonpolar cavity.²² Figure 2A shows that the cholesterol content of hippocampal membranes exhibits a progressive reduction upon

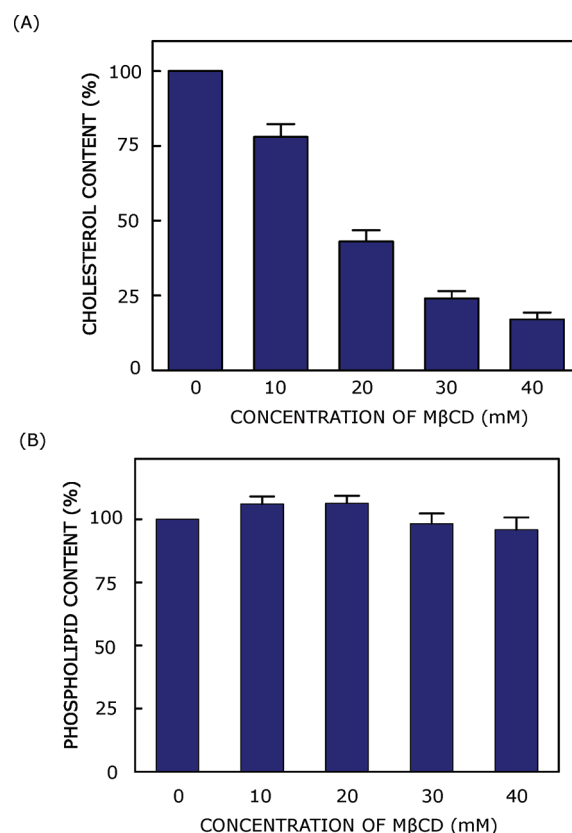


Figure 2. Lipid contents of hippocampal membranes upon cholesterol depletion. Hippocampal membranes were treated with increasing concentration of M β CD, followed by (A) cholesterol and (B) phospholipid estimation. Values are expressed as percentages of cholesterol and phospholipid contents in control (without M β CD treatment) of hippocampal membranes. Data represent means \pm SE of at least four independent measurements. See Experimental Section for other details.

treatment with increasing concentrations of M β CD. When hippocampal membranes were treated with 10 mM M β CD, cholesterol content was reduced to $\sim 78\%$ of the original value. This effect levels off at higher concentrations of M β CD, with the cholesterol concentration being reduced to $\sim 17\%$ of the original value when 40 mM M β CD was used (see Figure 2A). Importantly, there is no appreciable alteration in the membrane phospholipid levels under these conditions (see Figure 2B), thereby ensuring the specific nature of cholesterol removal.

In order to monitor the dynamic gradient in hippocampal membranes and its variation with cholesterol and protein content, we utilized spin-labeled phospholipids in which the spin label group (i.e., the paramagnetic nitroxide moiety) was positioned at different depths. While the spin label group is located at the membrane interface region in 5-PC (~ 12 Å from the center of the bilayer), the position of the label is much deeper in the hydrocarbon region of the membrane in the case of 14-PC (~ 4 Å from the center of the bilayer).^{33,34} ESR spectra of 5-PC incorporated into native hippocampal membranes (a) and cholesterol-depleted membranes (b, using 40 mM M β CD) are shown in Figure 3A. The spectra of 5-PC incorporated into liposomes of lipid extract from hippocampal membranes (c) and cholesterol-depleted hippocampal membranes (d, using 40 mM M β CD) are also shown in Figure 3A. Corresponding ESR spectra of 14-PC under similar conditions (e–h) are shown in Figure 3B. It is apparent from

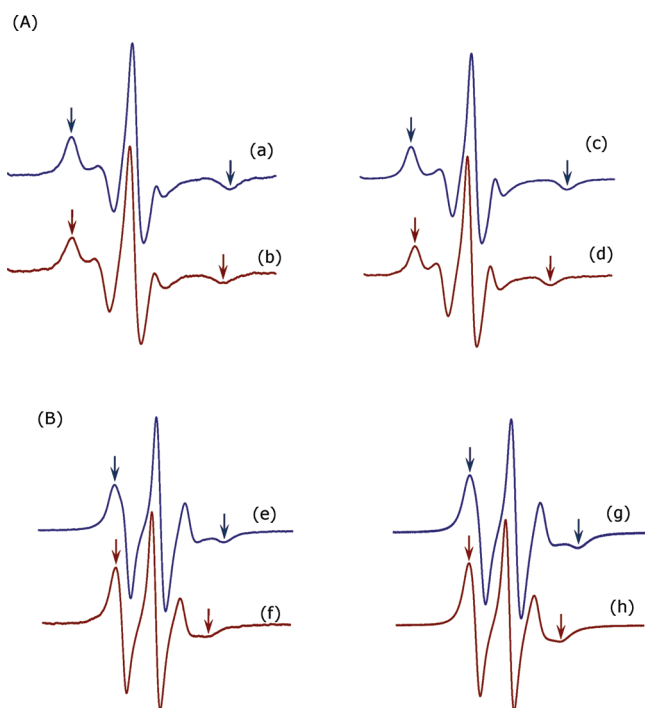


Figure 3. Typical ESR spectra of (A) 5-PC and (B) 14-PC incorporated in hippocampal membranes and liposomes of lipid extract. Spectra shown correspond to control (a and e) and cholesterol-depleted (b and f) hippocampal membranes. ESR spectra corresponding to liposomes of control (c and g) and cholesterol-depleted (d and h) lipid extract are also shown. Cholesterol depletion was carried out with 40 mM *M* β CD. See Experimental Section for other details.

the spectra that the positions of the first maximum and the last minimum display a slight shift toward each other upon cholesterol depletion in each case (indicated by arrows), although the overall shape of the spectra remains similar. This results in a reduction in outer maximum hyperfine splitting ($2A_{\text{max}}$) values for both 5- and 14-PC. The outer maximum hyperfine splitting is a sensitive parameter in ESR spectroscopy and contains information on motional dynamics and environmental polarity sensed by the spin label.^{45,46} While increase in motional dynamics results in a reduction in hyperfine splitting ($2A_{\text{max}}$), an increase in polarity leads to an increase in hyperfine splitting. The value of $2A_{\text{max}}$ exhibits a reduction from 59.0 to 56.2 G for native hippocampal membranes upon cholesterol depletion in the case of 5-PC (see panels a and b in Figure 3A). The corresponding reduction in $2A_{\text{max}}$ for liposomes of lipid extract is from 58.1 to 54.6 G (see panels c and d in Figure 3A). However, the value of $2A_{\text{max}}$ displays a reduction from 41.2 to 37.6 G for native membranes upon cholesterol depletion when 14-PC was used (see panels e and f in Figure 3B). The corresponding reduction in $2A_{\text{max}}$ in the case of liposomes of lipid extract is from 41.1 to 37.1 G (see panels g and h in Figure 3B) when 14-PC was used. The observed reduction in $2A_{\text{max}}$ values of 5- and 14-PC upon cholesterol depletion could have its origin to a combination of change in microenvironmental polarity due to altered water penetration and motional dynamics upon cholesterol depletion.^{26–29,47} We have previously shown, using pyrene vibronic band intensity ratio, that the apparent polarity of hippocampal membranes exhibits an increase upon cholesterol depletion.²⁹ This suggests that the observed reduction in $2A_{\text{max}}$ would have been larger if there

were no change in polarity. A comprehensive table of $2A_{\text{max}}$ values in native membranes and liposomes of lipid extract with progressive cholesterol depletion is shown in Supporting Information (see Table S2).

In order to analyze the observed changes in ESR spectra of the spin-labeled lipids under these conditions in a comprehensive and rigorous manner, we carried out spectral simulation of the observed ESR spectra obtained under these conditions using the NLLS approach. Analysis of ESR spectra of spin labels in such slow environments poses a considerable challenge since the relationship between ESR spectral features and physical parameters of interest is not direct. The NLLS approach provides a convenient and useful tool for the analysis of ESR spectra in a motionally restricted (slow) environment typically experienced by spin-labeled lipids in natural membranes.^{41–43,48} The NLLS analysis of ESR spectra of spin-labeled phospholipids with the nitroxide group attached to different positions (depths) in the acyl chain would provide valuable information on membrane order and dynamics in a depth-dependent manner. The best-fit of the simulated ESR spectra of 5- and 14-PC in native hippocampal membranes and cholesterol-depleted membranes are shown in Figure 4A,B. It is

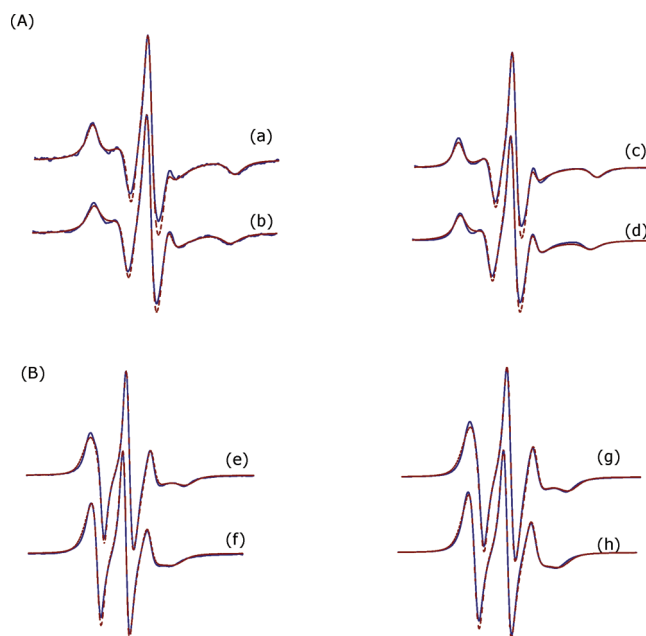


Figure 4. Typical ESR spectra of (A) 5-PC and (B) 14-PC incorporated in hippocampal membranes and liposomes of lipid extract (solid line). The simulations by NLLS analysis are also shown in each case (dotted line). Spectra and simulations are shown corresponding to control (a and e) and cholesterol-depleted (b and f) hippocampal membranes, and liposomes of control (c and g) and cholesterol-depleted (d and h) lipid extract. Cholesterol depletion was carried out with 40 mM *M* β CD. See Experimental Section for other details.

apparent from Figure 4 that the simulated spectra are in excellent agreement with the recorded spectra. The simulated spectra of 5- and 14-PC in hippocampal membranes and liposomes of lipid extract could be fitted well with a single component (simulations with two components produced inconsistent results). We interpret the simulation parameters as representative of the time-averaged values corresponding to dynamically heterogeneous lipid populations. Fits of compara-

ble quality were also achieved for ESR spectra of 5- and 14-PC in cholesterol-depleted conditions in hippocampal membranes and liposomes of lipid extract (see Figure 4). The parameters derived from the best-fit simulations corresponding to 5- and 14-PC incorporated in hippocampal membranes and liposomes of lipid extract are listed in Tables 1 and 2, respectively.

Table 1. NLLS Analysis of ESR Spectra in Hippocampal Membranes^a

spin label	M β CD (mM)	cholesterol content (%)	S_0	$R_L (\times 10^{-7})$ (s ⁻¹)
5-PC	0	100	0.37 \pm 0.03	7.44 \pm 0.02
	10	78	0.36 \pm 0.04	7.44 \pm 0.02
	20	43	0.35 \pm 0.07	7.41 \pm 0.05
	30	24	0.34 \pm 0.00	7.51 \pm 0.01
	40	17	0.34 \pm 0.00	7.53 \pm 0.01
14-PC	0	100	0.36 \pm 0.00	8.30 \pm 0.00
	10	78	0.34 \pm 0.01	8.25 \pm 0.04
	20	43	0.31 \pm 0.00	8.18 \pm 0.02
	30	24	0.29 \pm 0.00	8.15 \pm 0.02
	40	17	0.28 \pm 0.01	8.16 \pm 0.02

^aValues of the parameters shown correspond to means \pm standard deviations obtained from three independent measurements. All measurements were carried out at room temperature ($\sim 25^\circ\text{C}$). See text and Experimental Section for other details.

Table 2. NLLS Analysis of ESR Spectra in Liposomes of Lipid Extract^a

spin label	M β CD (mM)	cholesterol content (%)	S_0	$R_L (\times 10^{-7})$ (s ⁻¹)
5-PC	0	100	0.48 \pm 0.01	7.47 \pm 0.01
	10	78	0.46 \pm 0.02	7.53 \pm 0.01
	20	43	0.43 \pm 0.00	7.60 \pm 0.01
	30	24	0.42 \pm 0.02	7.65 \pm 0.02
	40	17	0.42 \pm 0.01	7.66 \pm 0.02
14-PC	0	100	0.37 \pm 0.02	8.51 \pm 0.03
	10	78	0.37 \pm 0.01	8.69 \pm 0.34
	20	43	0.32 \pm 0.00	8.44 \pm 0.03
	30	24	0.30 \pm 0.00	8.40 \pm 0.02
	40	17	0.27 \pm 0.01	8.37 \pm 0.03

^aValues of the parameters shown correspond to means \pm standard deviations obtained from three independent measurements. All measurements were carried out at room temperature ($\sim 25^\circ\text{C}$). See text and Experimental Section for other details.

Figure 5 shows the best-fit values of order parameter (S_0) of 5- and 14-PC in hippocampal membranes and liposomes of lipid extract. The order parameter exhibits progressive reduction with decreasing cholesterol content in all cases. The extent of reduction in the order parameter appears more pronounced in the case of liposomes of lipid extract. For example, the maximum reduction observed in the order parameter for hippocampal membranes is $\sim 8\%$ when 5-PC was used, while the corresponding value for liposomes of lipid extract is $\sim 13\%$. This overall trend is valid even when membrane order is monitored at a deeper location using 14-PC. The extents of reduction observed in the order parameter in this case are $\sim 22\%$ and $\sim 27\%$ for native membranes and liposomes of lipid extracts, respectively. This indicates that both native membranes and liposomes of lipid extract become disordered upon cholesterol depletion. These results are in agreement with previous reports utilizing fluorescence spectro-

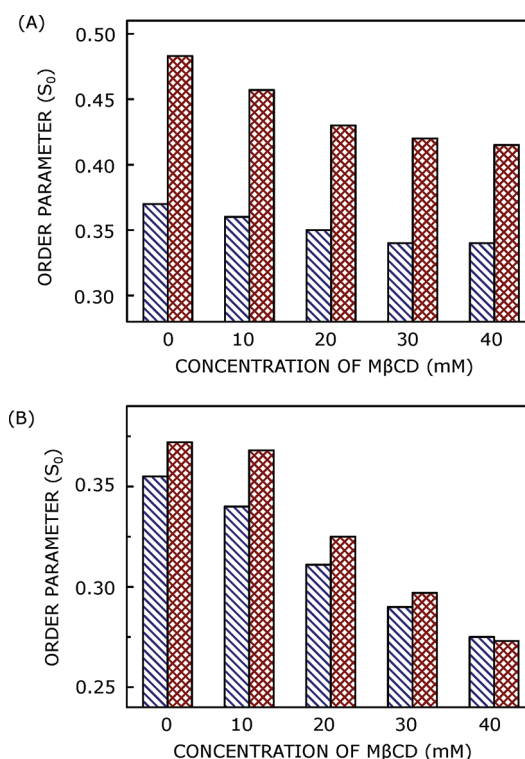


Figure 5. Order parameter (S_0) of (A) 5-PC and (B) 14-PC incorporated in hippocampal membranes (hatched bar) and liposomes of lipid extract (crisscrossed bar) upon increasing cholesterol depletion. The order parameter provides a measure of the angular extent of the rotational diffusion of the nitroxide moiety relative to the bilayer normal. See Tables 1 and 2 and Experimental Section for other details.

scopic approaches that cholesterol depletion leads to a reduction in membrane order in native hippocampal membranes.^{22,26} This implies that the reduction in membrane order is not limited to a particular time scale, but covers a broad range of time scales (\sim nanoseconds to microseconds). The observed depth-dependence of the extent of reduction in membrane order parameter upon cholesterol depletion merits comment. The change in the order parameter is considerably high when 14-PC was used to monitor membrane dynamics. These results imply that the deeper hydrocarbon region of the membrane is more sensitive to changes in membrane organization and dynamics due to cholesterol depletion than the interfacial region,^{49,50} in agreement with our previous results.²⁷

It is interesting to note that the order parameter of 5-PC in liposomes (0.48) is considerably higher compared to that of native membranes (0.37). This points out the difference in membrane packing (and therefore membrane order) in these two cases, possibly due to the bumpiness induced by membrane proteins ($\sim 75\%$ of total proteins in hippocampal membranes are estimated to be integral membrane proteins; data not shown). Similar observations have been made regarding the effect of proteins on the lipid acyl chain packing and order parameter of lipid spin labels incorporated into the plasma membranes of live RBL-2H3 mast cells as well as plasma membrane vesicles derived from them.^{48,51} Interestingly, this difference in order parameter between liposomes of lipid extract and native membranes is absent when 14-PC was used. This indicates that membrane proteins exert considerable influence on membrane order in the membrane interfacial

region. This effect decreases in deeper regions of the membrane, possibly due to the mobility gradient that exists along the length of the fatty acyl chain.⁵²

We report here an order parameter of 0.37 for 5-PC in native membranes at 25 °C (Figure 5A). This value is slightly lower than the order parameter obtained for 5-PC (0.42) in plasma membrane vesicles derived from the RBL-2H3 mast cells at 23 °C,⁵¹ and significantly lower than the order parameter of 5-PC in sphingomyelin model membranes at 20 °C in the gel phase (0.46) and in detergent-resistant membranes at 22 °C (0.52).^{51,53} Interestingly, the order parameter of 5-PC in native hippocampal membranes is comparable to the order parameter of 0.38 obtained for the same spin label in the liquid-ordered component of live RBL-2H3 cells at 25 °C.⁴⁸ This is in agreement with our previous results on native hippocampal membranes using fluorescence approaches. For example, we have previously reported that the fluorescence polarization of the popular membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH) in native hippocampal membranes is ~ 0.33 at 25 °C,²² a value that is characteristic of liquid-ordered phase in membranes.^{54,55} In addition, we earlier reported that native hippocampal membranes display a characteristic wavelength dependence of Laurdan generalized polarization (GP), reminiscent of the liquid-ordered phase.²⁶ The apparent liquid-ordered nature of native membranes could be attributed to high levels (~ 31 mol %) of cholesterol^{26,36} in hippocampal membranes since liquid-ordered phase membranes typically contain high amounts of cholesterol.^{14,55}

The best-fit values of the rotational diffusion coefficient (R_{\perp}) of 5- and 14-PC in hippocampal membranes and liposomes of lipid extract under different conditions are shown in Tables 1 and 2 and summarized in Figure 6. The value of R_{\perp} in liposomes is found to be higher compared to that of native membranes in all cases. It is also apparent from Figure 6 that R_{\perp} displays much less sensitivity under conditions of cholesterol depletion. We report here a value of $7.44 \times 10^7 \text{ s}^{-1}$ as R_{\perp} for 5-PC in native membranes at 25 °C (Figure 6A and Table 1). This value is clearly higher than the reported R_{\perp} of 5-PC in detergent-resistant membranes of RBL-2H3 cells at 22 °C ($4.07 \times 10^7 \text{ s}^{-1}$) and considerably higher than the corresponding value in sphingomyelin model membranes at 20 °C in the gel phase ($1.62 \times 10^7 \text{ s}^{-1}$).⁵³ The value of R_{\perp} of 5-PC in native membranes is comparable to the corresponding value ($7.41 \times 10^7 \text{ s}^{-1}$) in the less abundant liquid-crystalline-like component of plasma membrane vesicles derived from RBL-2H3 cells at 22 °C.⁵¹

Although the membrane lipid composition of bovine hippocampus is not known, the phospholipid composition of rat hippocampus has been reported.^{56–58} Analysis of the phospholipid composition of the rat hippocampus shows phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine as the predominant headgroups, while the fatty acid composition shows enrichment with 16:0, 18:0, 18:1, 18:2, 20:4, and 22:6 fatty acids. In addition, plasmalogens have been reported in rat hippocampus. In this work, we have monitored the organization and dynamics of hippocampal membranes and their modulation by cholesterol and protein content utilizing depth-specific spin-labeled phospholipids. Our results show that while cholesterol increases hippocampal membrane order, membrane proteins increase lipid dynamics resulting in disordered membranes by disturbing the membrane order (see Figure 7). It is noteworthy to mention here that these results are obtained using ESR spectroscopy that provides

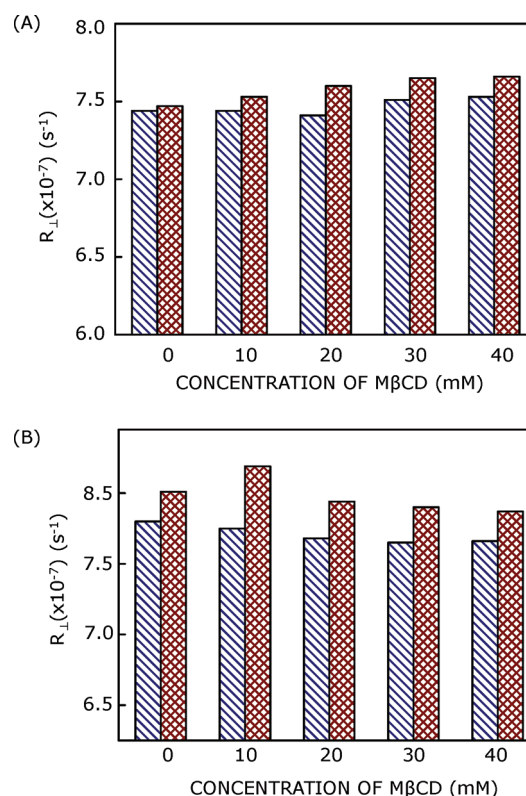


Figure 6. Rotational diffusion coefficient (R_{\perp}) of (A) 5-PC and (B) 14-PC incorporated in hippocampal membranes (hatched bar) and liposomes of lipid extract (crisscrossed bar) upon increasing cholesterol depletion. R_{\perp} represents the rotational diffusion coefficient of the nitroxide radical around the axis perpendicular to the mean symmetry axis for rotation. See Tables 1 and 2 and Experimental Section for other details.

information in a relatively slow time scale, appropriate for a slow diffusing system such as crowded neuronal membranes.^{30,31}

Knowledge of membrane order and dynamics would help in analyzing functional data generated by modulation of membrane lipid composition.^{4,22} The interaction between cholesterol and other molecular components in neuronal membranes (such as receptors and lipids) assumes relevance for understanding brain function. The organization and dynamics of cellular membranes in the nervous system is therefore significant for a comprehensive understanding of the functional roles played by the membrane-bound neuronal receptors which represent crucial components in signal transduction in the nervous system.

Taken together, our results constitute one of the first reports on depth-dependent changes in the organization and dynamics of hippocampal membranes and its modulation by cholesterol and protein content using ESR (characterized by relatively slow time scale) of depth-specific spin-labeled phospholipids. Membrane organization and dynamics represent important determinants in protein–protein interactions in cell membranes and have significant impact on the overall efficiency of the signal transduction process.^{59–61} Interestingly, membrane organization under low cholesterol conditions is relevant since reduced membrane cholesterol results in manifestation of several physiological effects. For example, it has been previously shown that cholesterol depletion affects sorting,⁶² distribution,⁶³ endocytosis,⁶⁴ and trafficking⁶⁵ of membrane proteins.

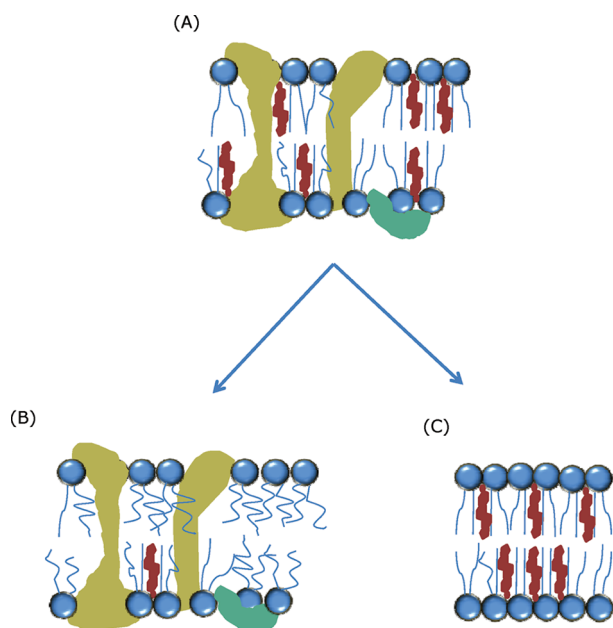


Figure 7. Schematic representation of the membrane organization in (A) native, (B) cholesterol-depleted, and (C) liposomes of lipid extract of hippocampal membranes. Phospholipids are shown in blue, cholesterol in maroon, and peripheral and integral membrane proteins in light green and mustard color, respectively. (A) The interaction between fatty acyl chains of phospholipids with the rigid sterol ring of cholesterol increases membrane order. (B) Cholesterol depletion increases the degree of segmental motion of fatty acyl chains of phospholipids leading to relatively less ordered membranes. (C) Increase in order accompanied by removal of membrane proteins: liposomes of lipid extract display increased order relative to native membranes under similar conditions. See Figure 5 and text for more details.

Importantly, we recently reported that chronic cholesterol depletion impairs the function of the serotonin_{1A} receptor, which could have important implications in mood disorders.²⁵ In a broader perspective, our results are significant in understanding the complex spatiotemporal organization of neuronal membranes and could have functional implications in neuronal diseases such as the Smith–Lemli–Opitz syndrome^{17,23,66} characterized by low cholesterol conditions due to defective cholesterol biosynthesis.

■ ASSOCIATED CONTENT

■ Supporting Information

Values of g and A tensor components used for simulations; outer maximum hyperfine splitting ($2A_{\text{max}}$) values of ESR spectra in hippocampal membranes and liposomes of lipid extract. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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