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Cholesterol-dependent thermotropic behavior and organization of neuronal membranes

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ABSTRACT

The composition of neuronal membranes is unique with diverse lipid composition due to evolutionary requirement. The organization and dynamics of neuronal membranes are crucial for efficient functioning of neuronal receptors. We have previously established hippocampal membranes as a convenient natural source for exploring lipid-protein interactions, and organization of neuronal receptors. Keeping in mind the pathophysiological role of neuronal cholesterol, in this work, we used differential scanning calorimetry (DSC) and small angle X-ray scattering (SAXS) to explore thermotropic phase behavior and organization (thickness) of hippocampal membranes under conditions of varying cholesterol content. Our results show that the apparent phase transition temperature of hippocampal membranes displays characteristic linear dependence on membrane cholesterol content. These results are in contrast to earlier results with binary lipid mixtures containing cholesterol where phase transition temperature was found to be not significantly dependent on cholesterol concentration. Interestingly, SAXS data showed that hippocampal membrane thickness remained more or less invariant, irrespective of cholesterol content. We believe that these results constitute one of the early reports on the thermotropic phase behavior and organizational characterization of hippocampal membranes under varying cholesterol content. These results could have implications in the functioning of neuronal receptors in healthy and diseased states.

1. Introduction

The nervous system is enriched with a diverse variety of lipids. The lipid diversity has been proposed to arise in response to evolutionary requirement of higher cognition in primates [1,2]. Cholesterol is an important lipid in the nervous system since it is known to affect neurotransmission by regulating the function of neuronal receptors [3–7], thereby giving rise to mood and anxiety disorders [8]. Brain cholesterol is implicated in a number of neurological disorders [9–11]. An interesting aspect of brain cholesterol is that it is exclusively synthesized *in situ* and there is no evidence of cholesterol transport from the blood plasma to the brain [12], at least in humans [13]. Defective cholesterol metabolism in the brain therefore results in a number of neurological disorders [14]. In this overall scenario, exploring neuronal membrane organization in the context of membrane cholesterol modulation assumes relevance.

In our laboratory, we have previously established bovine hippocampal membranes as a primary source for studying the interaction of membrane lipids with neuronal G protein-coupled receptors (GPCRs) such as the serotonin$_{1A}$ receptor [15,16]. A crucial finding from these studies is that cholesterol-induced membrane organization is necessary for the function of neuronal receptors [3,5–7,16]. With the overall objective to correlate cholesterol-dependent functional changes of neuronal receptors with alterations in membrane organization and dynamics, we previously utilized approaches based on fluorescence spectroscopy [17–20], and electron spin resonance spectroscopy [21]. Although spectroscopic approaches provide a wealth of information on the probe environment, the information obtained is local (short range) in nature.

Membrane phase is an important determinant of membrane organization and function [22,23]. Alteration of membrane cholesterol is often associated with changes in membrane phase [24–26] and thickness [27,28]. Differential scanning calorimetry (DSC) [29–31] and small angle X-ray scattering (SAXS) [32] are commonly used techniques to characterize thermotropic phase behavior, membrane organization and thickness in model and biological membranes. In this work, we used DSC and SAXS to probe the changes in thermotropic behavior and organization of hippocampal membranes under conditions of varying cholesterol content. Our results constitute one of the first reports on changes in thermotropic behavior and organization of

Abbreviations: BCA, bicinchoninic acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; GPCR, G protein-coupled receptor; MβCD, methyl-β-cyclodextrin; PMSF, phenylmethylsulfonyl fluoride; SAXS, small angle X-ray scattering; Tris, *tris*-(hydroxymethyl)aminomethane.

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hippocampal membranes with respect to cholesterol content which could provide novel insight into functional changes under these conditions.

2. Materials and methods

2.1. Materials

Cholesterol, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), methyl-β-cyclodextrin (MβCD), EDTA, EGTA, iodoacetamide, phenylmethylsulfonyl fluoride (PMSF), sucrose, sodium azide, Na_2HPO_4, 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/Invitrogen (Eugene, OR). 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 −80 °C till further use.

2.2. Methods

2.2.1. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously [16], flash frozen in liquid nitrogen and stored at −80 °C. Protein concentration was assayed using BCA reagent with bovine serum albumin as standard [33].

2.2.2. Cholesterol depletion from native hippocampal membranes

Native hippocampal membranes were depleted of cholesterol using MβCD as described previously [16,34]. Hippocampal membranes resuspended at a protein concentration of 2 mg/ml were treated with different concentrations of MβCD in 50 mM Tris buffer (pH 7.4) at room temperature (25 °C) with constant shaking for 1 h. Membranes were then spun down at 50,000 ×*g* for 5 min, washed once with 50 mM Tris buffer (pH 7.4) and resuspended in the same buffer. Cholesterol content was estimated using the Amplex Red cholesterol assay kit [35].

2.2.3. Estimation of phospholipids

Lipid phosphate was assayed subsequent to total digestion by perchloric acid using Na_2HPO_4 as standard [36]. DMPC was used as an internal standard to assess lipid digestion. Samples without perchloric acid digestion produced negligible readings.

2.2.4. Sample preparation

Native and cholesterol-depleted hippocampal membranes containing ~5–15 mg of total protein were suspended in 1 ml of 50 mM Tris buffer (pH 7.4) and used for DSC and SAXS experiments. Each sample was vortexed for 3 min before carrying out experiments.

2.2.5. Differential scanning calorimetry

Thermotropic behavior of native and cholesterol-depleted hippocampal membranes was investigated using a MicroCal VP-DSC microcalorimeter (Northampton, MA). Before running the DSC scan, each sample was degassed for ~10 min at 20 °C to avoid bubbles. All samples were subjected to two heating and two cooling scans between 1 and 110 °C at a scan rate of 1 °C/min. In each experiment, the first heating scan was considered for the determination of apparent phase transition temperature in the thermogram. Thermograms were overlaid to display the phase transition peaks and the overlaid plots were generated using Origin version 7.0 (OriginLab, Northampton, MA).

2.2.6. Small angle X-ray scattering

Scattering profiles of native and cholesterol-depleted hippocampal membranes were recorded for 1 h at 20 °C using S3 Micro (Hecus X-ray Systems GmbH, Graz, Austria). Scattering profiles were obtained using methods described previously [37]. The repeat distance or *d*-spacing of native and cholesterol-depleted hippocampal membranes were calculated using the following equation [38,39]:

$$d = 2\pi/q_{peak} \quad (1)$$

where *d* is the repeat distance (unit cell periodicity) and q_{peak} denotes the maximum of the scattering peak. Here, *d* represent the sum of the membrane bilayer (including membrane proteins) and water (hydration) layer thickness [39]. We calculated the average *d*-spacing of hippocampal membranes from the *d*-spacing values obtained from the first and second order scattering peaks.

3. Results and discussion

3.1. Change in cholesterol content in hippocampal membranes upon MβCD treatment

We have previously shown that MβCD, a water-soluble oligomer, acts as an efficient agent to selectively extract cholesterol from hippocampal membranes by including cholesterol in its central nonpolar cavity [16,40]. Fig. 1 shows that cholesterol content in hippocampal membranes progressively decreases upon treatment with increasing concentration of MβCD. Treatment with 10 mM MβCD was able to reduce cholesterol content to ~86% of the control membrane. The extent of cholesterol depletion increases with increase in MβCD concentration and was highest when 40 mM MβCD was used, where cholesterol content was reduced to ~26% of the control. Fig. 1 shows that the membrane phospholipid level remained mostly invariant upon MβCD treatment. The inset in Fig. 1 shows the corresponding values of cholesterol to phospholipid ratio (mol/mol) in hippocampal

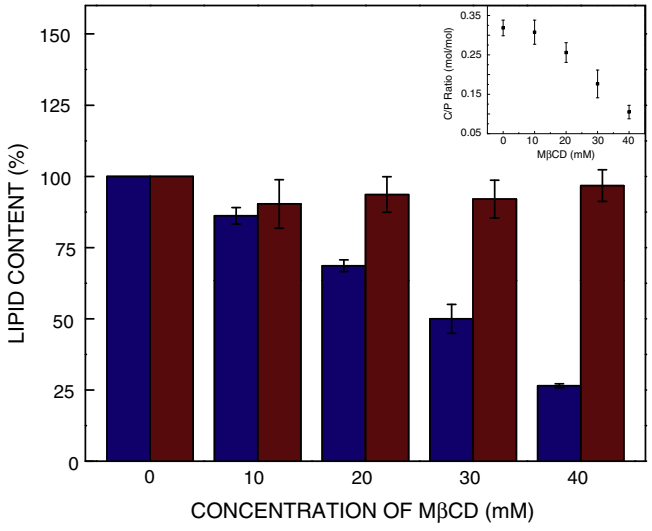

Fig. 1. Cholesterol (blue bars) and phospholipid (maroon bars) contents upon depletion of cholesterol in native hippocampal membranes with increasing concentration of MβCD. Values are expressed as percentages of the respective lipid content in native hippocampal membranes in the absence of MβCD. The inset shows the change in cholesterol to phospholipid ratio (C/P) (mol/mol) with increasing concentration of MβCD in hippocampal membranes. Data shown represent means ± S.E. of three independent experiments. See Materials and methods section for other details.

membranes under these conditions. As expected, a progressive reduction in cholesterol to phospholipid ratio was observed upon treatment with increasing concentration of MβCD.

Hippocampal membranes are neuronal in origin, and are rich in protein and cholesterol [41]. Previous results using a number of approaches that include fluorescence polarization of membrane probes [16], wavelength dependence of Laurdan generalized polarization (GP) [17], fluorescence properties of a Nile Red-based phase-sensitive membrane probe [20], and order parameters from electron spin resonance (ESR) spectra of spin-labeled phospholipids [21] have indicated the liquid-ordered nature of native hippocampal membranes. The liquid-ordered phase is characterized by extended (ordered) acyl chains (as in the gel phase), but exhibits high lateral mobility, similar to the liquid-disordered phase [42]. The liquid-ordered phase has been shown to exist above a threshold level of cholesterol for binary lipid mixtures [42]. The apparent liquid-ordered nature of hippocampal membranes could be attributed to the high cholesterol content (~30 mol%) in these membranes.

3.2. Cholesterol-dependent thermotropic phase behavior of hippocampal membranes

DSC thermograms of native and cholesterol-depleted hippocampal membranes are shown in Fig. 2a. The thermograms were overlaid to display multiple transition peaks. The thermogram of native hippocampal membranes exhibited multiple transition peaks with a prominent peak centered at ~29 °C (shown with an arrow in Fig. 2a). Multiple transition peaks in the thermogram could be due to lipid-lipid, lipid-protein and protein-protein interactions. Fig. 2b shows that the apparent phase transition of hippocampal membranes exhibits an interesting dependence on the extent of cholesterol depletion. The apparent phase transition temperature shows an increase from ~29 °C in native hippocampal membranes to ~31, ~34, ~38, and ~41 °C, in cholesterol-depleted hippocampal membranes obtained using 10, 20, 30 and 40 mM of MβCD, respectively (Fig. 2b).

Interestingly, we observed a single broad transition peak in the second heating thermogram, close to the major peak obtained in the first heating thermogram, and the remaining transitions were absent (see Fig. 3a). We interpret this difference between the first and second heating thermograms to be mostly due to the absence of protein contribution in the second heating thermogram, since proteins would undergo thermal denaturation at high temperatures [43]. The second heating

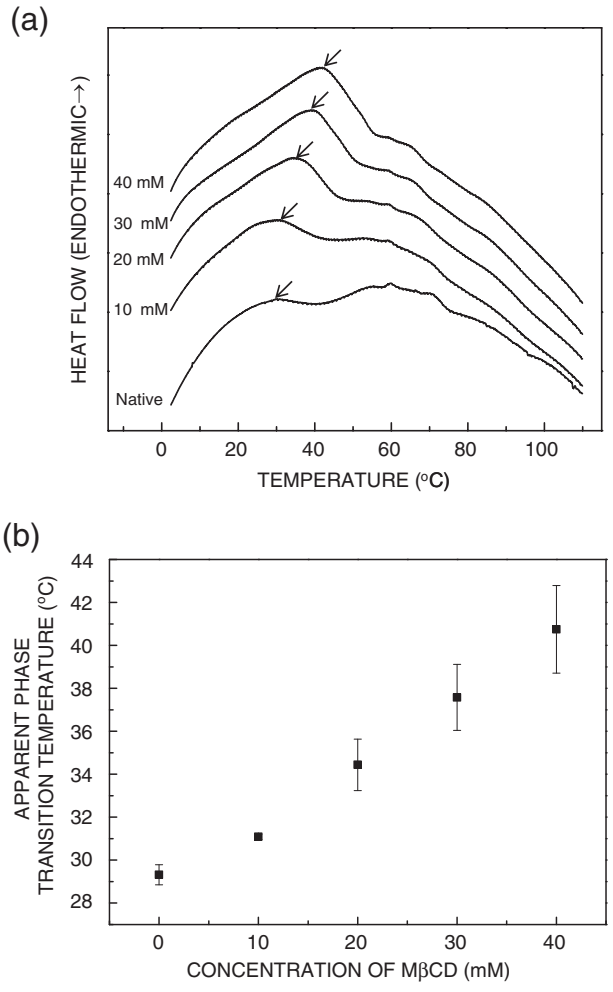

Fig. 2. (a) DSC first heating thermograms of native and cholesterol-depleted hippocampal membranes. The corresponding concentration of MβCD used is indicated against each thermogram. Thermograms were overlaid to display the apparent phase transition peaks (indicated by arrows). (b) Effect of MβCD on the apparent phase transition temperature of native and cholesterol-depleted hippocampal membranes. Apparent phase transition temperatures represent means ± S.E. of three independent experiments. See Materials and methods section for other details.

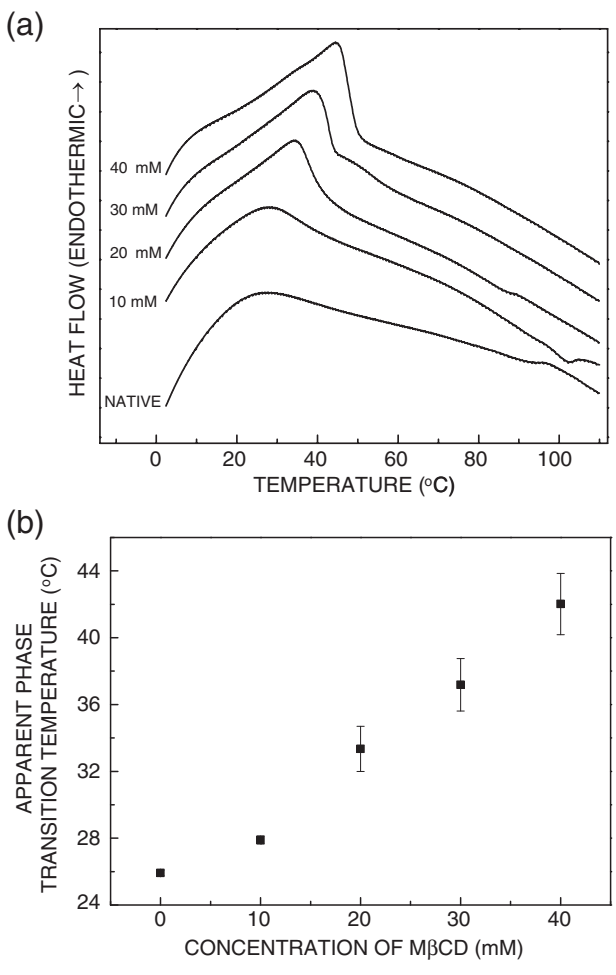

Fig. 3. (a) DSC second heating thermograms of native and cholesterol-depleted hippocampal membranes. The corresponding concentration of MβCD used is indicated against each thermogram. Thermograms were overlaid to display apparent phase transition peaks. (b) Effect of increasing concentration of MβCD on the apparent phase transition temperature, obtained from second heating thermograms of native and cholesterol-depleted hippocampal membranes. Data for apparent phase transition temperatures represent means ± S.E. of three independent experiments. See Materials and methods section for other details.

thermogram therefore mostly corresponds to transition peak due to hippocampal lipids. The second heating thermograms of cholesterol-depleted hippocampal membranes exhibited single transition peaks (see Fig. 3a). Based on this, we considered the major transition peak corresponding to ~29 °C in the first heating thermogram as the 'apparent phase transition peak' of native hippocampal membranes. Fig. 3b shows that the apparent phase transition temperature of hippocampal membranes exhibits continuous increase upon cholesterol depletion.

As mentioned above, thermograms of native and cholesterol-depleted membranes exhibited apparent phase transition peaks with varying concentrations of MβCD (see Fig. 2a). The apparent phase transition temperature exhibited a linear increase with increase in MβCD concentration (see Fig. 2b). Similar to the first heating thermogram, the apparent phase transition temperatures of native and cholesterol-depleted hippocampal membranes in the second heating thermogram exhibited a linear increase upon cholesterol depletion (see Fig. 3b). It is evident from these results that lipid phase transition peaks are reproducible when subjected to multiple heating scans, whereas a mixture of lipids and proteins could give rise to heterogeneity resulting in the appearance of multiple peaks in thermograms. The increase in the apparent phase transition temperature could be due to reduction in liquid-ordered phase of hippocampal membranes induced by cholesterol depletion, as shown previously by our group using a phase-sensitive fluorescent membrane probe [20].

3.3. Small angle X-ray scattering of native and cholesterol-depleted hippocampal membranes

In order to explore organization of hippocampal membranes under varying cholesterol conditions, we carried out SAXS measurements. SAXS profiles of native and cholesterol-depleted hippocampal membranes are shown in Fig. 4a. The scattering profiles of native and cholesterol-depleted hippocampal membranes exhibited a prominent first order scattering peak with the upper limit (q_{peak}) at 0.78 nm^{-1}, and a small second order scattering peak at 1.59 nm^{-1}. The corresponding *d*-spacing values were observed to be 80.5 Å and 79.0 Å, respectively. The average *d*-spacing was calculated from the *d*-spacing values obtained from the first and second order scattering peaks, and was found to be 79.3 Å for native hippocampal membranes (see Fig. 4b). The *d*-spacing values of hippocampal membranes calculated from our data are in excellent agreement with previously reported *d*-spacing values of rat synaptic plasma membranes [44]. The *d*-spacing value represents the sum of the membrane bilayer (including membrane proteins) and surface hydration layer thickness [39]. Interestingly, there was no appreciable difference in *d*-spacing values between native and cholesterol-depleted hippocampal membranes.

3.4. Correlation between apparent phase transition temperature and cholesterol content in hippocampal membranes

Since increasing extent of cholesterol depletion resulted in progressive increase of apparent phase transition temperature (Fig. 2b), we explored the correlation between phase transition temperature and membrane cholesterol content. For this, we plotted the apparent phase transition temperature (from Fig. 2b) as a function of cholesterol content in hippocampal membranes (from Fig. 1). This plot is shown in Fig. 5. Interestingly, a linear regression analysis between apparent phase transition temperature and hippocampal membrane cholesterol content resulted in a correlation coefficient (*r*) of ~0.99. The 99% confidence band, which contained all the data points, is plotted as dashed lines to indicate the significance of the close correlation (Fig. 5). Such a tight correlation indicates that apparent phase transition in neuronal membranes is dependent on membrane cholesterol content.

The phospholipid composition of the rat hippocampus has previously been analyzed. Phosphatidylethanolamine, phosphatidylcholine, and

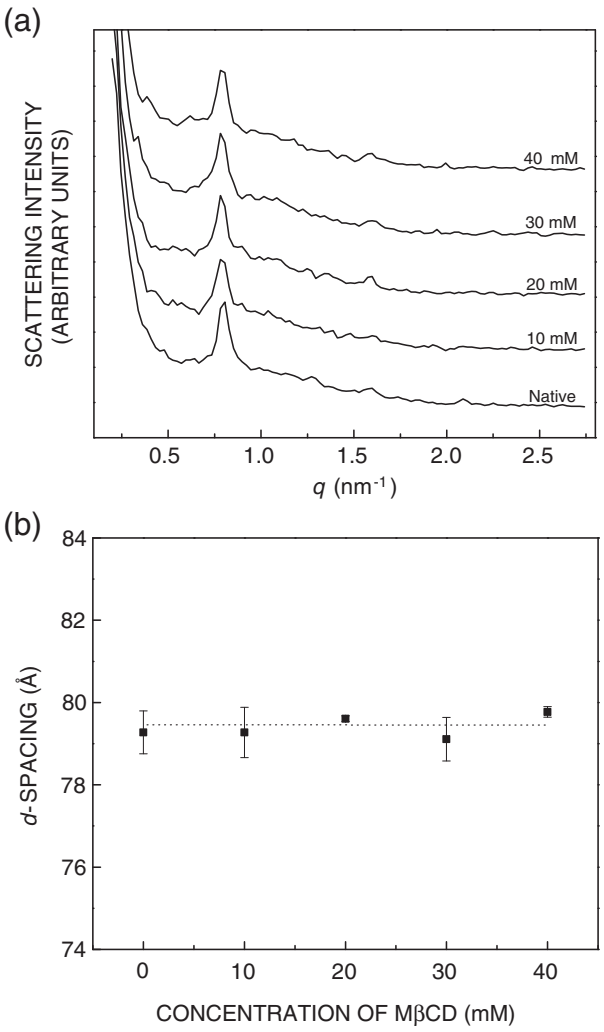

Fig. 4. (a) SAXS profiles of native and cholesterol-depleted hippocampal membranes. Corresponding MβCD concentrations are indicated against each scattering profile. SAXS profiles were overlaid to individually display scattering peaks. (b) The repeat *d*-spacing values of hippocampal membranes with increasing concentration of MβCD. The line joining the data points is provided merely as a viewing guide. Data represent means ± S.E. of three independent experiments. See Materials and methods section for other details.

phosphatidylserine are the predominant headgroups in rat hippocampus, while the fatty acid composition shows enrichment with 16:0, 18:0, 18:1, 18:2, 20:4, and 22:6 fatty acids [45–47]. In this work, we have examined the influence of cholesterol on the thermotropic phase behavior and organization of native and cholesterol-depleted hippocampal membranes using DSC and SAXS. Our results show that the apparent phase transition temperature of hippocampal membranes display a distinct dependence on cholesterol content. For example, the apparent phase transition temperature shows a considerable increase with decreasing membrane cholesterol content, with a ~41% increase when 74% cholesterol was depleted (Figs. 1 and 2). In addition, we show here that the apparent phase transition temperature is linearly and tightly correlated with hippocampal membrane cholesterol content (Fig. 5). This is an interesting observation in the context of thermotropic behavior of neuronal membranes. Previous work has shown that in a binary mixture of a saturated phospholipid and cholesterol, there is considerable broadening of the transition peak with little change in phase transition temperature [25,26,48]. In such systems, the phase transition temperature is not strongly correlated with cholesterol content in the membrane. In contrast to this, our results show that natural (neuronal) membranes exhibit a much pronounced dependence of apparent phase transition temperature on

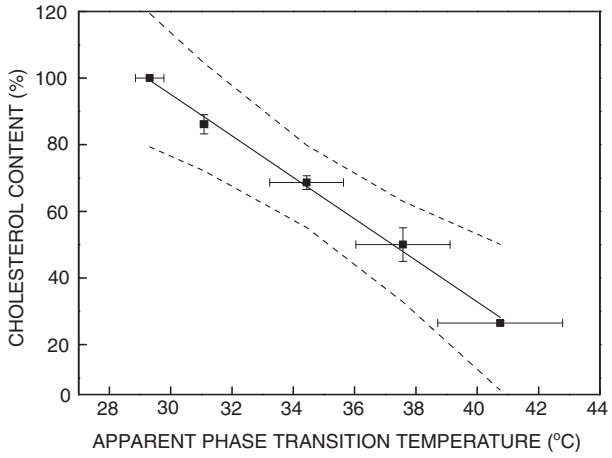

Fig. 5. Correlation of apparent phase transition temperature with cholesterol content in hippocampal membranes. Data plotted are taken from Figs. 1 and 2b. Linear regression analysis yielded a correlation coefficient ($r \sim 0.99$). The significance of the correlation is apparent from the 99% confidence band (plotted as dashed lines). The close correlation between apparent phase transition temperature and membrane cholesterol content is noteworthy. See text for more details.

cholesterol content. To the best of our knowledge, this type of cholesterol-dependent phase transition in functional neuronal membranes constitutes a novel observation. The physiological relevance of cholesterol-dependent thermotropic behavior of neuronal membranes, in terms of membrane organization and function, could provide valuable insight.

Our SAXS results show that the thickness of hippocampal membranes is not sensitive to changes in cholesterol content. This is a bit surprising since cholesterol has been reported to increase membrane thickness [28]. However, it has been reported that cholesterol has very little effect on thickness of cellular organelle membranes [49]. The relative insensitivity of hippocampal membrane thickness to cholesterol could be attributed to the presence of large amounts of proteins in these membranes that could lead to compensatory reorganization upon cholesterol depletion. We plan to address this issue in our future experiments by removing the proteins from hippocampal membranes, thereby making a total lipid extract.

Taken together, our results constitute one of the first reports on the thermotropic and organizational characterization of hippocampal membranes with varying cholesterol content using DSC and SAXS. We have previously shown that the activity of neurotransmitter receptors such as the serotonin$_{1A}$ receptor, a natural resident GPCR in hippocampal membranes, is inhibited upon cholesterol depletion [16,50]. The thermotropic phase behavior of hippocampal membranes reported in this work could have implications in functional modulation of neurotransmitter receptors in cholesterol-rich neuronal membranes. The possible effect of such cholesterol-dependent membrane changes in diseases such as the Smith–Lemli–Opitz syndrome [14], characterized by low cholesterol condition due to defective cholesterol biosynthesis and inhibition of neurotransmitter receptor activity [51], would be interesting to conjecture.

Conflict of interest

The authors declare that there is no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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