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Cholesterol Asymmetry in Synaptic Plasma Membranes

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Abstract

Lipids are essential for the structural and functional integrity of membranes. Membrane lipids are not randomly distributed but are localized in different domains. A common characteristic of these membrane domains is their association with cholesterol. Lipid rafts and caveolae are examples of cholesterol enriched domains, which have attracted keen interest. However, two other important cholesterol domains are the exofacial and cytofacial leaflets of the plasma membrane. The two leaflets that make up the bilayer differ in their fluidity, electrical charge, lipid distribution, and active sites of certain proteins. The synaptic plasma membrane (SPM) cytofacial leaflet contains over 85% of the total SPM cholesterol as compared with the exofacial leaflet. This asymmetric distribution of cholesterol is not fixed or immobile but can be modified by different conditions *in vivo*: 1) chronic ethanol consumption; 2) statins; 3) aging; and 4) apoE isoform. Several potential candidates have been proposed as mechanisms involved in regulation of SPM cholesterol asymmetry: apoE, low-density-lipoprotein receptor, sterol carrier protein-2, fatty acid binding proteins, polyunsaturated fatty acids, p-glycoprotein and caveolin-1. This review examines cholesterol asymmetry in SPM, potential mechanisms of regulation and impact on membrane structure and function.

Keywords

aging; apolipoprotein E; asymmetry; caveolin; cholesterol; lipid domains

There is substantial interest in membrane lipid domains across numerous areas of biology. The roles of lipid domains in brain structure, function and neurodegeneration are certainly one of those areas as demonstrated by this special issue. Rafts and caveolae are domains that have attracted considerable attention. However, two other domains of the membrane are actually the two leaflets of the bilayer as pictured in Figure 1. There are substantial differences in the two leaflets including for example electrical charge, thickness, fluidity, and lipid distribution (Schroeder 1985;Wood *et al.* 2002). Lipids are asymmetrically distributed in the membrane bilayer (Fig. 1). Phosphatidylcholine (PC) and sphingomyelin (SM) are enriched in the brain exofacial or outer leaflet of the synaptic plasma membrane. There is evidence that SM is not present in the cytofacial or inner leaflet of SPM but that phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) are in abundance in the cytofacial leaflet. This transbilayer or asymmetric distribution of phospholipids contributes to the differences in the electrical charges of the two leaflets. The exofacial leaflet is neutral or zwitterionic and the cytofacial leaflet is more negatively charged due to the enrichment of the anionic phospholipids, PI and PS. This difference in the electrical charge of the two leaflets is associated with the accumulation of certain

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cationic and anionic drugs in membranes (Sweet *et al.* 1987). Cationic drugs acted on the cytofacial leaflet and anionic drugs affected the exofacial leaflet. Regulation of phospholipid asymmetry is thought to involve the actions of different protein translocases and transporters and that topic has been recently reviewed (Bevers and Williamson 2010). Cholesterol, a major lipid in membranes accounting for over 40 mol% of synaptic plasma membrane lipids (Wood *et al.* 1989a) is also asymmetrically distributed. The purpose of this review is to discuss cholesterol asymmetry in brain synaptic plasma membranes (SPM), its alteration under certain conditions, mechanisms involved in its regulation and the role of cholesterol asymmetry in membrane structure and function.

Cholesterol Asymmetry in Plasma Membranes

The establishment of cholesterol asymmetry in biological membranes was advanced by the earlier work of Schroeder and colleagues using quenching of the fluorescent sterol dehydroergosterol (DHE) by trinitrobenzene sulfonic acid (TNBS). A comprehensive review on DHE has been recently published (McIntosh *et al.* 2008) and so a detailed discussion will not be presented in the present review. Briefly, DHE is a natural fluorescent sterol found in sponge and yeast and thus does not have a bulky fluorophore attached to it as compared for example with the commonly used cholesterol analog NBD-cholesterol. DHE is structurally and functionally most similar to cholesterol as compared with other cholesterol analogs. This fluorescent sterol is used in cell culture, isolated tissue, real-time imaging in living cells and administered *in vivo*. A caveat to using DHE is that the commercial compound is chemically synthesized and it can contain impurities such as oxidized sterols which can perturb membrane structure and function necessitating steps to remove such contaminants (McIntosh *et al.* 2008).

The mouse cytofacial leaflet of SPM contains substantially more cholesterol as compared with the exofacial leaflet (Igbavboa *et al.* 1997;Igbavboa *et al.* 1996;Wood *et al.* 1990;Kirsch *et al.* 2003). The cytofacial leaflet contains approximately 85% of total SPM cholesterol. That the cytofacial leaflet contains more cholesterol than the exofacial leaflet was also observed in fibroblasts (Incerpi *et al.* 1992), human erythrocytes (Schroeder *et al.* 1991) and most recently in the plasma membrane and the endocytic recycling compartment of a Chinese hamster ovary cell line (Mondal *et al.* 2009). Those findings indicate that cholesterol asymmetry is a property shared by different cell types with the abundance of cholesterol contained in the cytofacial leaflet. The greater concentration of cholesterol in the cytofacial leaflet versus the exofacial leaflet is associated with the fluidity of the two leaflets. The SPM cytofacial leaflet is distinctly less fluid than the exofacial leaflet (Wood *et al.* 2002). The large difference in leaflet fluidity affects the ability of various molecules to partition into membranes. For example, ethanol disorders the exofacial leaflet but has little if any effect on the cytofacial leaflet (Schroeder *et al.* 1988;Wood *et al.* 1989b;Bae *et al.* 2005).

SPM cholesterol asymmetry is not static and it is altered by chronic ethanol consumption, statins, aging and apolipoprotein E isoform. There was approximately a two-fold increase in cholesterol in the exofacial leaflet of mice chronically administered ethanol (Wood *et al.* 1990). The total amount of SPM cholesterol (exofacial leaflet + cytofacial leaflet) was similar for the ethanol and control groups. Not surprisingly, the fluidity of the two leaflets in the ethanol group was altered. The exofacial leaflet became less fluid and the cytofacial leaflet became more fluid in SPM of the ethanol group. This change in fluidity was consistent with the ethanol-induced redistribution of cholesterol between the two leaflets. Chronic administration of statins (simvastatin, lovastatin, atorvastatin) altered cholesterol asymmetry in mouse SPM (Burns *et al.* 2006). There was an increase in exofacial leaflet cholesterol and a corresponding reduction in cytofacial leaflet cholesterol. Those results are

different from the findings of an earlier study which showed that lovastatin and pravastatin but not simvastatin reduced cholesterol in the SPM exofacial leaflets of chronic drug-treated mice (Kirsch *et al.* 2003).

Increasing age alters SPM cholesterol asymmetry (Igbavboa *et al.* 1996). Mice 24–25 months of age had significantly more cholesterol in the SPM exofacial leaflet (32% cholesterol) as compared with mice 3–4 months of age (14% cholesterol). Mice 14–15 months of age also had significantly more cholesterol in the exofacial leaflet (24%) than the younger age group. In young mice, the exofacial leaflet is significantly more fluid than the cytofacial leaflet. This asymmetry in fluidity was not observed in SPM of aged mice, which may be due in part to the redistribution of cholesterol between the two leaflets. However, other factors must also contribute to the loss of differences in fluidity between the two leaflets of aged mice. Attenuation of cholesterol asymmetry has also been observed in SPM of mice expressing human apolipoprotein E4 (Hayashi *et al.* 2002). Both increasing age and the apoE4 allele are risk factors for Alzheimer's disease (AD) and changes in cholesterol asymmetry may contribute to the pathophysiology associated with AD. An active area of research has been on the association between cholesterol abundance and the production of the amyloid beta-protein (Aβ) including the role of lipid rafts and this topic is reviewed elsewhere in this issue. A two-fold increase or greater of cholesterol in the exofacial leaflet observed in SPM of aged mice and mice expressing human apoE4 could certainly impact on membrane structure and function and contribute to Aβ production. What makes changes in cholesterol asymmetry observed in aged mice or mice expressing human apoE4 so notable are that major alterations can occur in the absence of total changes in SPM cholesterol abundance.

Regulation of Membrane Cholesterol Asymmetry

The abundance of cholesterol in the exofacial leaflet is strikingly less as compared with the cytofacial leaflet. An explanation for cholesterol asymmetry has not been established. There have been several potential candidates proposed as mechanisms involved in regulation of SPM cholesterol asymmetry as depicted in Figure 2. Sphingomyelin had been proposed earlier to be a factor in cholesterol asymmetry (Slotte and Bierman 1988;Porn *et al.* 1991). Hydrolysis of sphingomyelin in fibroblasts and Leydig tumor cells caused movement of cholesterol from the cell surface to the cell interior. Sphingomyelin accounts for approximately 2 to 4% of the total non-sterol SPM lipid and it is all contained in the exofacial leaflet (Wood *et al.* 1993;Rao *et al.* 1993). In erythrocytes, sphingomyelin is approximately 25% of total non-sterol lipid and abundance in the exofacial leaflet was between 82 and 100 % (Roelofsen 1982). Erythrocyte exofacial leaflet cholesterol is approximately 25% of total membrane cholesterol (Schroeder *et al.* 1991). Increasing sphingomyelin levels in the exofacial leaflet is associated with increasing cholesterol content in that leaflet. Regulation of cholesterol in the exofacial leaflet but not the cytofacial leaflet may involve interaction of cholesterol and sphingomyelin via binding, complex formation, or changes in membrane structure such as fluidity and lipid packing. In addition, sphingomyelin is a component of lipid rafts and the influence of lipid rafts on cholesterol asymmetry and vice versa are topics that have not been examined.

There is evidence that both apoE and one of its receptors, the low density lipoprotein receptor (LDLR) may contribute to the maintenance of cholesterol asymmetry. SPM of mice deficient in apoE had a two-fold increase in exofacial leaflet cholesterol as compared with wild type mice (Igbavboa *et al.* 1997). This large difference cannot be accounted for by changes in the total amount of SPM cholesterol, which were similar in SPM of both groups. It was observed in the same study that mice deficient in the LDLR or deficient in both apoE and LDLR also showed greater abundance of cholesterol in the exofacial leaflet as compared

with wild type mice. ApoE is the major cholesterol transporter in brain, it is primarily synthesized in astrocytes and it has been shown that neurons receive some of their cholesterol from astrocytes (Mauch *et al.* 2001). Cholesterol in neurons is unique in contrast to phospholipids because it would appear that it is not synthesized at the nerve terminal of the axon (Vance *et al.* 1994). The nerve terminal including the SPM may receive some astrocyte derived cholesterol, which is delivered by apoE and taken up by LDLR and other family members. This cholesterol may then be recycled to SPM. A problem with this interpretation is that the SPM of the apoE and LDLR deficient mice either singly or the double-knockout had levels of total SPM cholesterol which were similar to wildtype mice. This observation does not support a deficit in transporting cholesterol from astrocytes to the nerve terminal.

Assuming that apoE and LDLR may contribute to the maintenance of cholesterol asymmetry, the changes in the null mice did not exceed 34% of cholesterol in the exofacial leaflet. This finding would imply that additional factors are involved in regulating cholesterol asymmetry. There are data indicating that fatty acid composition may be contributors to cholesterol asymmetry in plasma membranes. Plasma membranes of L-cell fibroblasts which were fed serum enriched in unsaturated fatty acids had approximately 70% of cholesterol sequestered in the exofacial leaflet as compared with 28% in the control exofacial leaflet (Sweet and Schroeder 1988). Linking fatty acid composition with SPM cholesterol asymmetry are data showing that SPM of apoE-deficient mice was enriched in the highly unsaturated fatty acid 22:6 (*n* & − 3) in both the sn-1 and sn-2 positions particularly in diacyl-PE and PS (Igbavboa *et al.* 2002). PE and PS are in abundance in the cytofacial leaflet and an increase in the phospholipid molecular species containing 22:6 (*n* & − 3) may stimulate the transbilayer movement of cholesterol directly or act on a putative protein that regulates cholesterol asymmetry. For example, it was shown that fibroblasts overexpressing live fatty acid binding protein (L-FABP) had more cholesterol in the exofacial leaflet as compared to control cells (Woodford *et al.* 1993). L-FABP is a cytosolic protein that binds both fatty acids and cholesterol (Schroeder *et al.* 2008).

Two additional proteins that could play a role in maintaining membrane cholesterol asymmetry are p-glycoprotein (P-gp) and caveolin-1 (Garrigues *et al.* 2002;Igbavboa *et al.* 2009). P-gp is a member of the ATP-binding cassette transporter family of proteins having multiple functions including multidrug resistance in certain types of tumor cells (Schinkel 1997). P-gp is expressed in brain (Spector 2010). It was reported that P-gp stimulated the movement of cholesterol from the cytofacial leaflet to the exofacial leaflet in vesicles prepared from DC-3F cells overexpressing human P-gp using accessibility of cholesterol to cholesterol oxidase to determine cholesterol distribution (Garrigues *et al.* 2002). This translocation of cholesterol was inhibited by a P-gp inhibitor. It also was concluded in that study that the cytofacial leaflet contained more cholesterol as compared with the exofacial leaflet, which is consistent with findings in other cells types using an entirely different method (DHE fluorescence) for determining cholesterol asymmetry as discussed earlier in this review. In that paper, it was proposed that P-gp might possibly interact with caveolin-1 in increasing exofacial leaflet cholesterol. There are data showing that P-gp co-immunoprecipitates with caveolin-1 (Demeule *et al.* 2000). Caveolin-1 is a 22-KDa protein associated with caveolae and this protein binds cholesterol and is thought to be a key contributor to cholesterol homeostasis (Murata *et al.* 1995;Smart *et al.* 1994;Conrad *et al.* 1995;Uittenbogaard and Smart 2000;Pol *et al.* 2001;Ito *et al.* 2002). We have recently reported that perturbation of astrocytes by amyloid beta protein (1–42) induced movement of cholesterol and caveloin-1 from the plasma membrane to the Golgi complex (Igbavboa *et al.* 2009). Effects of Aβ1-42 on both cholesterol and caveolin-1 were inhibited by siRNA targeted to the caveolin-1 gene. There was also a significant reduction of cholesterol and caveolin in the Golgi complex of cells treated with only siRNA. There is evidence that

caveolin may recycle lipids including cholesterol. One possibility is that cholesterol cycles in and out of the cytofacial leaflet and that caveolin may regulate cholesterol specifically in the cytofacial leaflet. Excess cholesterol in the cytofacial leaflet may be transported by caveolin or P-gp to the Golgi complex and other organelles. Caveolin may have a transbilayer effect and cycle cholesterol between the cytofacial and exofacial leaflet; similar to proteins involved in maintaining phospholipid asymmetry. A recent study found that caveolin-1 was enriched in the cytofacial leaflet and it sequestered fatty acids (Simard *et al.* 2010). As mentioned earlier (Sweet and Schroeder 1988), treating cells with unsaturated fatty acids altered cholesterol asymmetry and perhaps such changes could involve caveolin-1 complexing with fatty acids.

An obvious conclusion regarding regulation of cholesterol asymmetry is that a single mechanism does not appear to account for the greater abundance of cholesterol in the cytofacial leaflet as compared with the exofacial leaflet. Instead, we hypothesize that multiple mechanisms are involved which may include both proteins and lipids in regulating the transbilayer distribution of cholesterol.

Cholesterol Asymmetry and Membrane Function

It is well-established that cholesterol plays a major role in both membrane structure and protein function (Yeagle 1989;Schroeder *et al.* 2010;Levitan *et al.* 2010). How specific changes in the distribution of cholesterol in the two leaflets affect membrane function have not been extensively studied. There is some evidence that plasma membrane functions such as receptor-effector coupling, ion transporters, and translocation of proteins across the plasma membrane may be influenced by the transbilayer lipid environment including cholesterol (Schroeder *et al.* 2001). Export of cholesterol out of the cell to lipoprotein acceptors may be altered by changes in cholesterol asymmetry (Mondal *et al.* 2009). It has been reported that statin-induced redistribution of cholesterol was associated with reduced Aβ and β-CTF levels in contrast to changes in bulk cholesterol levels in brain membranes (Burns *et al.* 2006). SPM of chronic ethanol treated mice, which showed a doubling of cholesterol in the exofacial leaflet, were resistant to perturbation by ethanol indicative of neuronal tolerance. Changes in cholesterol asymmetry could impact on the capacity of the membrane to form domains such as lipid rafts and caveolae. Lipid and protein composition of lipid rafts from mice expressing human apoE4 differed from mice expressing human apoE3 (Igbavboa *et al.* 2005) and as discussed earlier apoE expressing mice had a greater percentage of SPM cholesterol in the exofacial leaflet as compared with apoE3 mice (Hayashi *et al.* 2002). What is not evident is whether changes in cholesterol asymmetry alters lipid rafts or in fact, lipid rafts contribute to the transbilayer distribution of cholesterol. Finally, a question, which has not been rigorously addressed, is if changes in cholesterol asymmetry are adaptive or conversely are such changes inimical to cell membrane function. The argument could be made that the changes observed in ethanol-treated mice may be adaptive i.e., reducing partitioning of ethanol into the membrane but changes in cholesterol asymmetry in SPM of aged mice or mice expressing human apoE4 which were similar to that of ethanol treated mice may not be adaptive. In the instance of the ethanol-treated mice, it is reasonable to predict that additional effects because of changes in cholesterol asymmetry would be observed which might not be adaptive. It is clear that much more research is needed to establish the functional consequences of modifying the transbilayer distribution of cholesterol in membranes.

Summary

Cholesterol is asymmetrically distributed in plasma membranes including SPM. The cytofacial leaflet contains approximately 5 to 6 fold more cholesterol than the exofacial

leaflet, which has both structural and functional consequences. Cholesterol asymmetry is not static but is altered by several different conditions both *in vivo* and *in vitro*. Mechanisms regulating cholesterol asymmetry are not well-understood but the available data lead to the conclusion that multiple mechanisms may be involved. The functional consequences of changes in SPM cholesterol asymmetry include fluidity, alterations in lipid domains, lateral and transbilayer diffusion, lipid packing, and protein function. Of particular interest and the need for further research is the relationship between cholesterol asymmetry and the formation and function of lipid rafts and caveolae.

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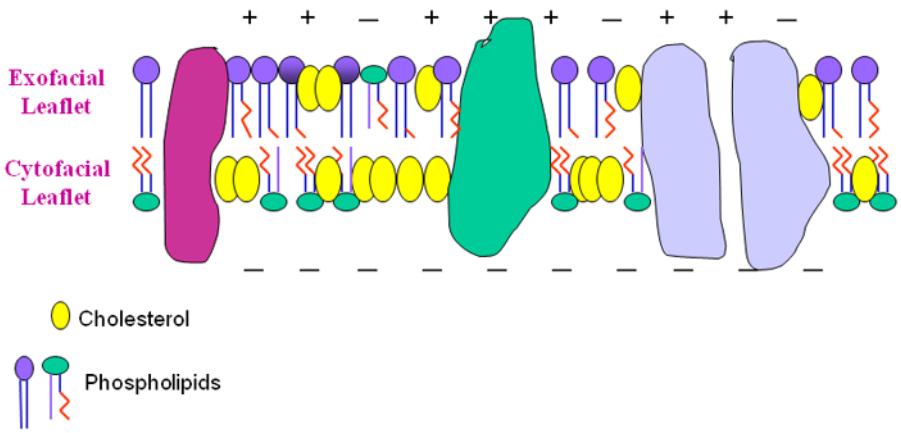

Figure 1.
Asymmetry in Synaptic Plasma Membranes. Model of the two leaflets of the plasma membrane, showing the asymmetric distribution of cholesterol and phospholipids. Large globular structures represent proteins. Cholesterol, phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine are enriched in the cytofacial leaflet. Phosphatidylcholine and sphingomyelin are in abundance in the exofacial leaflet, and there is some phosphatidylcholine in the cytofacial leaflet. + and − denote charge properties of the two leaflets.

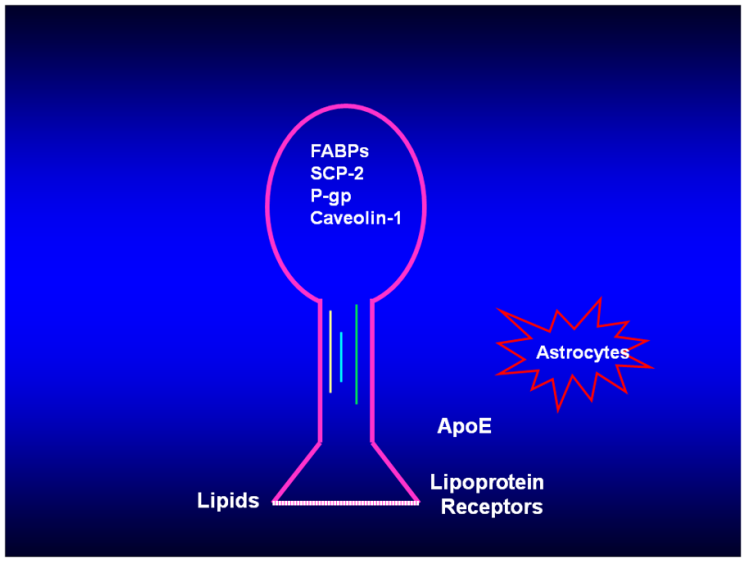

Figure 2.

Proposed Mechanisms Involved in Regulation of Cholesterol Asymmetry in Synaptic Plasma Membranes. Neuronal model containing intracellular proteins, which may transport cholesterol to or anchor cholesterol in the two leaflets of the plasma membrane. Cholesterol may also be transported from astrocytes to the nerve terminal and taken up by members of the low density lipoprotein receptor family. Fatty acid binding proteins (FABPs), sterol carrier protein-2 (SCP-2), P-glycoprotein (P-gp).