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Chapter

Lipid Rafts and Development of Alzheimer's Disease

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Abstract

A wealth of evidence accumulated over the last two decades has unambiguously linked lipid rafts to neurodegenerative diseases, in particular to Alzheimer's disease (AD). These microdomains are highly dynamic membrane platforms with differentiated physicochemical and molecular properties compared to the surrounding membrane microenvironment, and are the locus for a number of central processes in neuronal physiology. Most recent evidence pinpoint to lipid rafts as main players in AD neuropathology. It is now widely accepted that lipid rafts actively participate in the processing of amyloid precursor protein to generate amyloid beta peptides, a main component of amyloid plaques. Current evidence have highlighted the existence of severe alterations in the molecular structure and functionality of lipid rafts in the frontal cortex of human brains affected by Alzheimer's disease. An exceptionally interesting observation is that lipid raft destabilization can be demonstrated even at the earliest stages of AD neuropathology. In the present review, we will first elaborate on the structure and function of these multifaceted subcellular structures and second to focus on the impact of their alterations in neuronal pathophysiology along the onset and progression of AD continuum.

Keywords: membrane microdomains, lipid rafts, membrane neurochemistry, lipid-protein interactions, lipid raft biophysics, lipid raft aging, neurodegeneration, Alzheimer's disease (AD)

1. Lipid rafts: definition and significance

Our current view of cell membranes is far from the historical view of a being floating mixture of lipids and proteins mixed uniformly in the form of bilayers. Instead, evidence accumulated in the last three decades has revealed that membrane constituents can segregate to form discrete domains. The heterogeneous structures of membrane lipids provide them the ability to mix non-randomly in the bilayer and to form specific lipid microdomains. The best characterized class of these structural entities has been termed 'lipid rafts' which are featured by their higher contents of cholesterol and sphingolipids compared to their surroundings. Despite some controversies on a proper definition for lipid rafts, in 2006, at the Keystone Symposium on Lipid Rafts and Cell Function, it was agreed that "membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol, and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein-lipid interactions" [1].

Lipid rafts have been found in most cell types, from epithelial cells to neurons, and share essential chemical and physical properties, but differ in specific components, mainly proteins, which are responsible for functional heterogeneity of cell types, populations or even developmental stages.

The importance of lipid rafts in nerve cells lays in the fact that they behave as functional platforms which participate in a number of physiological processes involved in signal transduction, such modulation of receptor activities, protein interactions in transduction cascades, and the function of ion channels, but also in dendritic and axonal protein trafficking and sorting, regulation of neurotransmitter receptors and in the exocytotic neurotransmitter release, posttranslational modifications of proteins and lipids, and in many aspects related to cell-to-cell communication, including multifaceted synaptic physiology [2–4].

The agreement exist that these microdomains are highly dynamic structures providing transient and fluid architectural scaffolding platforms, which by undergoing structural and functional changes they accomplish a variety of functions in a coordinated intracellular and extracellular context. Remarkably, current evidence demonstrate they lipid rafts may also play significant roles in different pathological conditions. Thus, lipid rafts and raft components are key players in a variety of pathological events, i.e. by facilitating conversion of prion protein (PrP^{c}) to its infectious scrapie form (PrP^{sc}) [5], by regulation of Amyloid Precursor Protein processing in Alzheimer's disease [6], by expressing binding sites for toxins internalization such cholera toxin [7] or by providing specific entry pathways for various types of viruses and budding of mature virions from infected cells [8, 9] including the HIV-1 or the SARS-CoV-2 which is driving us mad, towards an unprecedented global chaos (by providing attachment of S-protein to ACE2 and other auxiliary proteins clustered in lipid rafts) [9], amongst other pathological processes. During the last decade, investigation on lipid rafts biology has received enormous attention due to the demonstration of its involvement in neurodegenerative diseases, in particular in Alzheimer's disease, as we will discuss later.

2. Biochemical and biophysical structure of lipid rafts

Besides being enriched in cholesterol and sphingolipids, lipid rafts are also endowed with a particular lipid signature, which makes them different from other domains in the non-raft membrane plane. Such differences are illustrated in **Figure 1** for membrane raft and non-raft fractions in the gray matter of human frontal cortex. As can be observed, compared to bulk non-raft membranes, lipid rafts contain higher contents of saturated fatty acids, lower levels of mono- and polyunsaturated fatty acids, and nearly all of the cellular contents of sphingomyelin, cerebrosides and sulfatides.

According to the polar head group, sphingolipids are divided in two major phosphosphingolipids such sphingomyelin, and glycosphingolipids which includes gangliosides, cerebrosides and sulfatides. Ceramide serves as the backbone to generate sphingolipids to produce sphingomyelin or more complex glycosphingolipids after incorporation of phosphocholine or sugars at the hydroxyl group. Both classes of sphingolipids, phosphosphingolipids and glycosphingolipids, are major components of lipid rafts and display pleiotropic behaviors affecting a number of essential functions associated with normal and pathological states, particularly in AD [10–12].

Gangliosides are acidic glycosphingolipids containing one or more sialic acid residues, representing about 6% of total lipids, and particularly abundant in raft-like lipid microdomains of neuronal cells [12, 13]. They are concentrated in the outer leaflet of the plasma membrane (**Figure 2**), where they are anchored by the

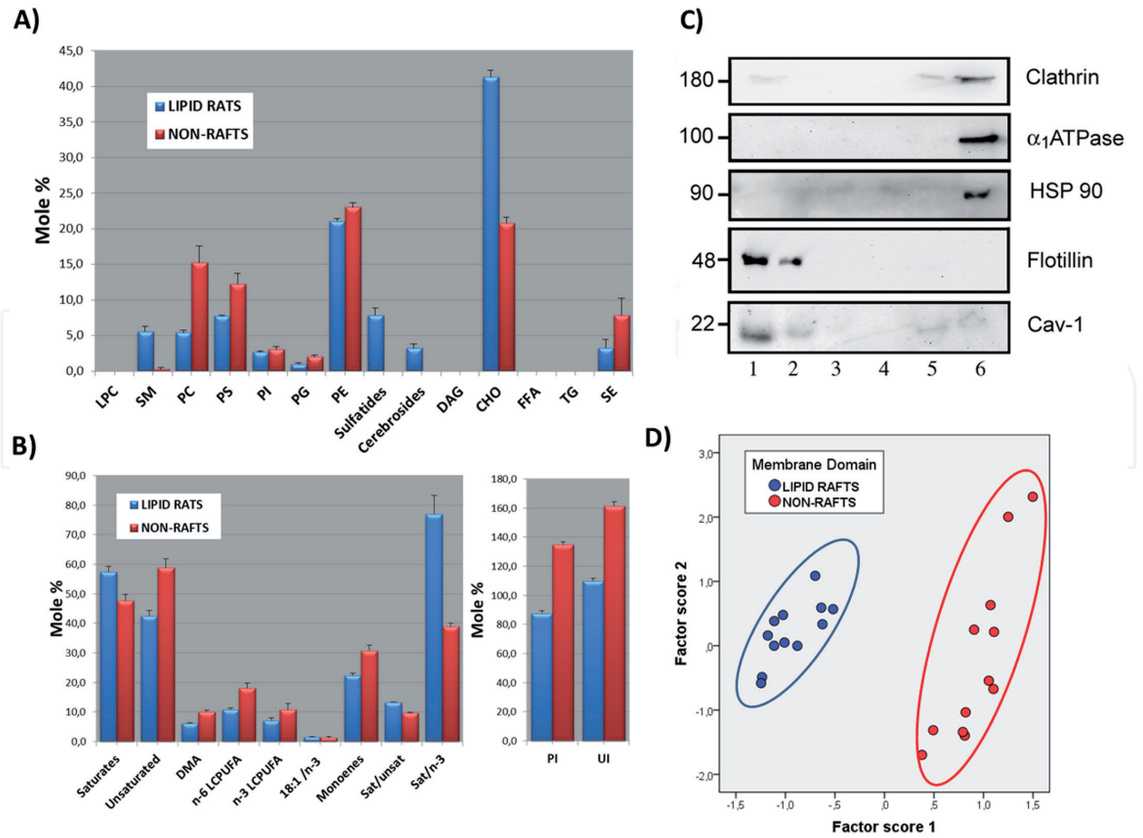

Figure 1.
Comparative analyses of lipid rafts and non-raft domain in the gray matter of human frontal cortex. (A) Lipid classes. LPC: Lysophosphatidylcholine, SM: Sphingomyelin, PC: Phosphatidylcholine, PS: Phosphatidylserine, PI: Phosphatidylinositol, PG: Phosphatidylglycerol, PE: Phosphatidylethanolamine, DAG: Diacylgycerides, CHO: Cholesterol, FFA: Free fatty acids, TG: Triacylglycerides, SE: Sterol esters. (B) Main fatty acid groups. DMA: Dimethylacetals, LCPUFA: Long-chain polyunsaturated fatty acids, PI: Peroxidability index, UI: Unsaturation index. (C) Distribution of protein markers in fractional separation of cell membranes. Fractions 1 and 2 correspond to lipid rafts while fraction 6 is mainly composed by non-raft membranes. (D) Score plots of lipids (fatty acids) resulting from multivariate analyses on membrane domains from mouse cortex illustrating the lipid fingerprints of raft and non-raft domains.

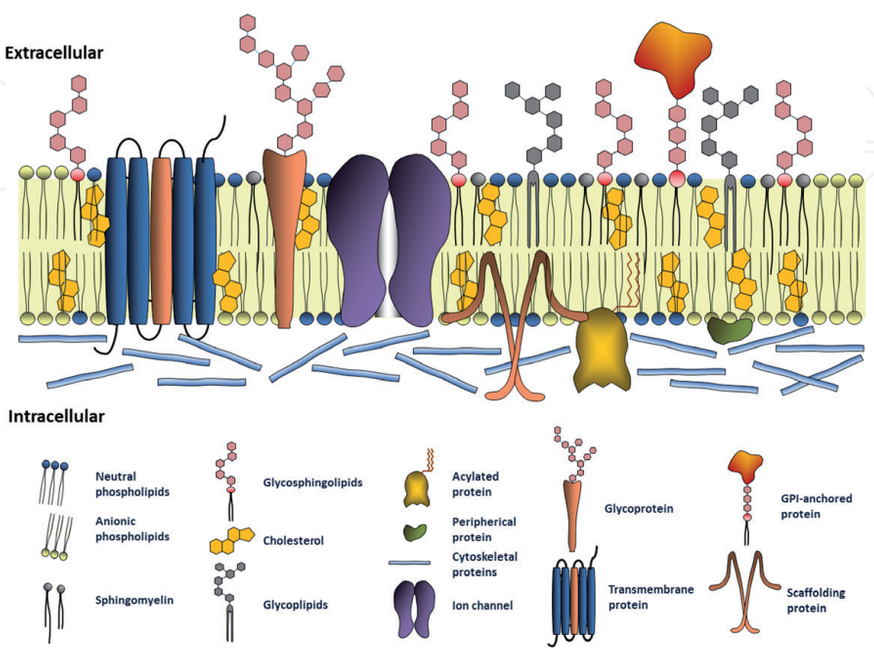

Figure 2.
Schematic representation of the lipid raft membrane structure. For details see the text.

hydrophobic ceramide part of their molecule while the oligosaccharide chain protrudes into the extracellular medium. Main gangliosides in the brain are GM1 and GDs (GD1a in a-series, and GD1b and GT1b in b-series) [11]. They participate in the two-dimensional and transverse structuration of the membrane, lipid–protein interactions and organization of lipid rafts [13, 14]. The high heterogeneity of oligosaccharide structures in gangliosides allows specific interactions with a diversity of molecules at the surface of cell membrane [10, 13, 14]. "Cis" and "trans" interactions of gangliosides play multiple roles in infectious diseases [15] where they act as cellular receptors and coreceptors for viruses, bacteria, and microbial toxins. Prominent examples are GM1 as the receptor for *Vibrio cholerae* toxin (cholera toxin), for *Clostridium botulinum* toxin (botulinum toxin), and for the SabA adhesin of *Helicobacter pylori* [15, 16]. Further, as we will discuss later, gangliosides are important regulators of amyloid β toxicity in Alzheimer's disease by modulation of polymerization of peptide species [17].

Lipid rafts biogenesis occurs in the trans-Golgi network, where their composition is set and the resulting vesicles fused to the plasma membrane. One remarkable characteristic lipid profile of lipid rafts in nerve cells is that, with the exception of gangliosides, sulfatides and sphingolipids, most lipid classes and fatty acids are present in significant amounts in raft and non-raft domains, but they differ substantially in their relative contents (**Figure 1A**). Triglycerides and free fatty acids are totally excluded from either domain. As a general rule, sphingolipids are more abundant in lipid rafts fraction and glycerophospholipids (or phospholipids) are more abundant in non-rafts fractions (**Figure 1A**). Amongst phospholipids, neutral phospholipids, phosphatidylethanolamine (PE) is the most abundant phospholipids in lipid rafts, while phosphatidylcholine (PC) and anionic phospholipids phosphatidylserine (PS) and phosphatidylinositol (PI) are present in significant proportion though less abundant than in non-raft domains. The presence of anionic phospholipids is paramount for neuronal physiology as they serve as sources for intracellular messengers and bioactive lipid mediators, i.e. inositol phosphates, diacylglycerol, eicosanoids (such prostaglandins and leukotrienes) and docosanoids (such Neuroprotectin D1) [17–22]. Fatty acids are also heterogeneously distributed between rafts and non-rafts, with saturates containing acyl chains of 16 or more carbon atoms being particularly abundant in lipid rafts. Monounsaturated (monoenes) and polyunsaturated fatty acids of the n-3 (mainly docosahexaenoic acid, DHA) and n-6 (mainly arachidonic acid, AA) series, are present at significantly lower amounts compared to non-rafts (**Figure 1B**).

DHA and AA are essential components of nerve cells membranes which esterify the sn-2 position of glycerophospholipids (mainly phosphatidylethanolamine and phosphatidylserine, the most abundant phospholipids in nerve cells). In cerebral gray and white matter, phosphoglyceride classes PE, PC, PS, PG and PI have distinctive LCPUFA profiles. Thus, ARA greatly exceeds DHA in phosphatidylinositol, whereas DHA exceeds ARA in phosphatidylserine. Brain phospholipids also include plasmalogens, which contain a vinyl-ether and an ester bond at the sn-1 and sn-2 positions, respectively [23]. As with conventional phospholipids, plasmalogens are classified according to their head group in the sn-3 position, the most abundant plasmalogens being plasmenyl-ethanolamine (PlsEtn) and plasmenyl-choline (PlsCho). The sn-2 position of PlsEtn and PlsCho display preferential esterification by LCPUFAs, a fact whereby plasmalogens are considered important LCPUFAs reservoirs of in nerve membranes.

Overall, differences in the lipid fingerprint of raft and non-raft domains (**Figure 1A** and **B**) are sufficiently different as to allow the complete discrimination of membrane domains based on a multivariate approach (**Figure 1D**). It turns out that saturated long acyl chains of phospholipids and especially sphingolipids, allow

tight intermolecular packing through hydrophobic interactions within the bilayer, providing differentiated lipid complexes in juxtaposition with kinked unsaturated acyl chains of bulk membrane phospholipids. Raft lipids are held together by relatively weak non-covalent bonds, establishing a dynamic equilibrium of raft and non-raft regions in the plasma membrane. In the case of sphingolipids, these molecules interact laterally through van der Waals interactions and hydrogen bonds between their sphingosine backbones. Further, as the majority of sphingolipids contain long saturated acyl chains, their tighter intramembrane packing with associated lipids allows the formation of stable gel–liquid phase which lead to laterally segregation of sphingolipid-rich domains from their glycerophospholipid-rich surroundings [24, 25]. Structurally, this degree of lateral association is further increased by the incorporation of cholesterol, whose planar sterol ring interacts with the saturated acyl chains [26].

One key difference between phospholipids and sphingolipids is the length and saturation of their acyl chains. These acyl chains are always saturated and longer in sphingolipids than in phospholipids and allow hydrophobic interactions between the two leaflets of the bilayer [27, 28]. These molecular attributes are directly implicated not only in the formation of domains enriched in sphingolipids, but also in the coupling between the two leaflets in the rafts by interdigitation of the very long chain fatty acid between exoplasmic and cytoplasmic leaflets (**Figure 2**) and by augmenting hydrogen bonding in sphingolipid-sterol rich domains [28, 29]. This particularity is very important because it implies that lipid rafts exist as stable bilayer structures [28]. Hydrophilic interactions between phospholipid head groups are also critical as they provide physical forces for raft stability and formation of lipid shelves.

In physical terms, the more dense islands of sphyngolipid-, cholesterol- and saturated-rich domains, representing lipid rafts, exist in a liquid-ordered state (*'lo'* phase). It is widely accepted that rafts exist in nerve cell membranes in the liquid-ordered phase display limited lateral and rotational mobility in the bilayer. Cholesterol molecules intercalate filling gaps in sphingolipid packing, and increases the rigidity and molecular density of bilayers in lipid rafts due to its ability to tightly pack with saturated lipids when the *lo* phase is formed [30, 31]. The surrounding phospholipid bilayer enriched in unsaturated acyl chains exist in a state termed 'liquid-crystalline' or 'liquid-disordered, *ld*', and represent non-raft domains, in which the lipid acyl chains are fluid and disordered, exhibit much higher intermolecular mobility. The degree of disorder within this *ld* phase in nerve cells is considerable, as they contain the largest amount of polyunsaturated fatty acids (n-3 ad n-6 series) in the whole organism. Phospholipid-rich sphingolipid-poor liquid-crystalline domains (non-rafts) and sphingolipid- and cholesterol-rich liquid-ordered phase domains (rafts) exist in dynamic equilibrium in biological membranes [31, 32].

The fact that lipid rafts are in an ordered *lo* phase provides them an extremely useful property for technical purposes: they are resistant to solubilization in the cold by nonionic detergents (such as Triton X-100) and therefore can be isolated by differential ultracentrifugation as 'detergent-resistant membranes' or DRM (referring to the physical structure isolated by detergent insolubility, while the term 'raft' refer to the microdomain in the intact membranes). This has allowed the identification of proteins and lipids which display preferential (or exclusive) partitioning into rafts [32] (**Figure 1C**).

A number of proteins have been found associated to DRM and the list of candidates is steadily growing [33]. The term 'raftophilic' has been coined to refer to the preferential location of these proteins in DRM or lipid rafts (**Figure 1C**). Recently, a database (RaftProt), containing more than 47,000 entries (V2.0, 2020 version)

of putative raftophilic proteins identified in mass spectrometry studies of isolated DRMs has been published [34]. Many of these proteins are not prototypical transmembrane proteins but display post-translational modifications aimed at favoring their targeting to lipid rafts (**Figure 2**). The first family of proteins described is GPI-anchored proteins. This family of proteins is anchored to the outer leaflet of the membrane through covalent attachment to a special glycolipid, glycosyl phosphatidylinositol (GPI) [35]. Amongst the GPI-anchored proteins involved in neuronal physiology, one of the best characterized is the cellular prion protein (PrP^c) [36]. PrP^c is constitutively expressed in neurons and preferentially localized in lipid rafts. PrPc is known to play different physiological roles in nerve cells, including regulation of ion channels and neurotransmitter receptors at the pre- and postsynaptic levels [37] and has been linked to the pathogenesis of prion disease as mentioned before [36]. Prion disease is characterized by the conformational modification of normal PrP^c into a misfolded and aggregated abnormal conformer, the pathogenic infectious form PrP^{sc}, [38]. Current evidence indicates that conversion into PrP^{sc} is entirely dependent on the lipid raft microenvironment [38].

Other raft-associated proteins are linked to saturated acyl chains through biochemical processes grouped as lipidation [39] (**Figure 2**). Lipidation is particularly important for membrane binding of peripheral membrane proteins (though it may also occur in transmembrane proteins). Often these proteins are directly acylated in specific residues with two or more palmitate chains, or a palmitate and a myristate chain. These lipid modifications, named S-palmitoylation and N-myristoylation, are finely regulated and determine not only the fate of modified proteins to target lipid rafts, but also contribute to their stabilization within the domain and modulate protein interactions occurring within rafts. Such post-translational modifications are commonly found in Src family of tyrosine kinases (STKs) [40] and scaffolding proteins [41]. Prenylation of proteins is also a lipidation mode for membrane association, consisting on a covalent attachment of an isoprenoid chain (either farnesyl- or geranyl-) to the C-terminus of proteins favoring their membrane association. This type of modification are common between members of the small G-proteins family, including Ras and Rab proteins involved in cellular signaling and oncogenicity [39, 42].

Common hallmark proteins of lipid rafts are caveolins and flotillins (**Figure 2**). These raft-resident proteins act as scaffolding structures within these microdomains [43, 44]. It should be mention that caveolin family was first known for its participation in the formation of caveolae, membrane invaginations involved in endocytosis and signaling commonly observed in non-neuronal cell types such endothelial or epithelial cells [45]. Soon after, caveolin-1 was shown to display high affinity for rafts, and to be consistently extracted in DRMs. Though these two families of scaffold proteins are not transmembrane proteins, they undergo palmitoylation, allowing their anchoring to the cytoplasmic leaftet of the bilayer, and have the intrinsic capacity to form lipid shells around themselves [43]. Most evidence suggests that the lipid-modified nature of these scaffolds proteins integrated in lipid rafts serve not only to aid targeting them to these domains but also to stabilize rafts themselves. In line with this, caveolin-1 tends to form high-molecular-weight oligomers which associate with each other in the plane of the membrane [44]. Further, numerous studies have concluded that these scaffold proteins help to compartmentalize specific signaling molecules within lipid rafts, and to modulate the specificity of protein interactions, with the final prospect of rapidly and selectively modulating cell signaling events [28, 46]. In this sense, the presence of caveolin-binding motifs in many raft proteins allow them to bind to the scaffolding domain of caveolin, which serve as a molecular filter to gather related signaling proteins close to each other, and to support additional protein–protein

interactions [47]. In the case of flotillins, an evolutionarily conserved domain named "prohibitin homology domain" (PHB) determines the affinity for flotillins and the raftophilic nature of proteins carrying it [48]. These properties are crucial for the formation of dynamic multimolecular platforms termed signalosomes, with complex functions in normal and pathological nerve cells.

3. Lipid rafts in neuronal cell signaling

Current evidence demonstrate that neuronal lipid rafts serve as docking platforms that bring together a number of specific proteins which determine the specificity of neuronal functioning and communication. They include different families of proteins with functions as receptors, ion channels, transporters, membrane-bound enzymes, signaling proteins, interacting proteins, molecular adaptors, amongst others. They all share a special ability to interact with surrounding lipids mainly through lipid modifications, such lipidation with lipophilic anchors (*S*-palmitoylation and *N*-myristoylation, prenylation, GPI-anchoring) or to cholesterol itself or by specific domains in their secondary structure to facilitate their integration in lipid rafts, such cholesterol recognition amino acid consensus (CRAC motifs) [49] and phospholipid binding sites [50]. In general, the integration of a protein in the raft membrane initiates interaction with surrounding proteins within multimolecular complexes or signalosomes which are dynamically recruited to lipid rafts in response to specific stimuli. They are believed to rearrange into large, stable membrane rafts, and to associate to downstream signaling molecules when bound to cognate ligands, activating signalosomes, and eventually triggering specific biochemical events involved in the many facets of neuronal physiology [2–4, 32].

One of the best studied multimolecular complexes in neurons is membrane rafts in postsynaptic neurons, which along with PSDs (postsynaptic densities), are considered major sites of synaptic signaling [3, 51]. In depth proteomics analyses performed by [52] in PSD have allowed identification of a number of proteins (>150) in PSD-included lipid rafts which are exclusive for postsynaptic membrane rafts, and not shared by non-raft portions of PSD. Most of these proteins could be classified as typical raft proteins (i.e. flotillin-1 and 2, PrP^c), cell adhesion molecules (i.e. contactin, cadherin), ion channels (i.e. voltage-dependent calcium channels, inwardly rectifying potassium channels, NGF-gated Ca^{2+} channels), transporters (i.e. facilitated glucose transporter, high affinity glutamate transporter, GABA transporter protein, H^+-ATPase), kinases/phosphodiesterases (i.e. Ca^{2+}/calmodulin-dependent protein kinase II, phosphodiesterase 2A, cGMP-stimulated) and G-proteins/small G-proteins (i.e. heterotrimeric G-protein subunits, members of RAS oncogene family) [52, 53].

The scenario emerges that, at least in PSD, the high protein density in raft membranes creates a crowded environment in which lipid–lipid packing is affected by proteins, probably with a stronger effect inside the more ordered raft-like domains [54]. Even more, it could be envisaged that this dense packing would limit intradomain mobility and thereby affecting protein interactions and conformational changes. To this author, this is one of the principal reasons to explain the evolutionary selection of significant amounts of highly unsaturated long chain fatty acids (LCPUFA) in nerve cells lipid rafts, which we have consistently found in brain raft preparations from different origins [55, 56]. Indeed, brain tissue contains the largest amount of LCPUFA in the whole body, well above the adipose tissue, and more importantly, they are contained exclusively in cell membranes. Most frequent LCPUFA in nerve cells are docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6), whose acyl chains contain 6 and 4 double bonds, respectively.

The amounts of these fatty acids in neuronal membranes differ between rafts and non-rafts domains, the later containing 3-4 times more LCPUFA than raft membranes [57, 58]. Even so, the degree of polyunsaturation of lipid rafts provides a sufficient degree of fluidity in the *lo* domain of lipid rafts, and a physical mechanism to ensure lipid and protein movements, such lateral and rotational diffusional rates as well as conformational displacements, required for proper intermolecular interactions [59, 60]. The regular bends introduced by the double bonds in the acyl chain of DHA and AA limit the stiffness of the packed bilayer and confer raft proteins a degree of motion freedom enough to accomplish molecular interactions. It is worth mentioning that, on a molar base, the contribution of unsaturation to bilayer fluidity is much higher for LCPUFA than for monounsaturated fatty acids, the most important in brain membranes being oleic acid (OA, 18:1n-9). In neural lipid rafts, this effect of LCPUFA on difussional rates is amplified by the unfavorable and repulsive interactions between the high cholesterol levels and polyunsaturated phospholipids [54, 61]. Overall, the lipid scenario in nerve cell lipid rafts renders them less ordered than in similar domains from non-neural cells. Importantly, destabilization of this physicochemical property of neural lipid rafts underlie dramatic consequences in lipid raft functioning in Alzheimer's disease, as we will discuss in next sections.

Perhaps largest evidence demonstrating the significance of lipid rafts in nerve cell function is neurotrophic factor signaling. Most receptors for neurotrophic factors are receptor tyrosine kinases (RTKs) residing (or recruited to) lipid rafts which are activated upon binding to the specific trophic factor and undergo activation autophosphorylation of specific tyrosine residues. Activated RTKs are docking proteins for multimolecular complexes or signalosomes that activate downstream intracellular signaling cascades through molecular adaptors which are also raftphilic. Final effectors of RTK signaling are keys for regulation synaptic transmission, differentiation, axon guidance and cell adhesion [2, 62].

Receptor tyrosine kinases observed to reside in lipid rafts include tropomyosin-related kinase A (TrkA) receptor and the low-affinity p75 neurotrophin receptor (p75NTR), which are receptors for Nerve Growth Factor (NGF) [63], IGF-1R (insulin growth factor-1 receptor) [64], EGFR (epidermal growth factor receptor) [65] or PDGFR (platelet-derived growth factor receptor) [66, 67], amongst others. Alternatively, RTKs that are not lipid rafts resident proteins, may translocate to rafts after activation, as it was initially demonstrated for glial-derived neurotrophic factor (GDNF)-mediated activation of the Ret RTK [2].

The Src family of protein tyrosine kinases (STKs) is composed by integral raft proteins widely expressed in the CNS and are particularly abundant in neurons. Src is the best studied STK, is ubiquitous but in nerve cells are expressed as different isoforms in a neuron-specific mode. It has been reported that at least five SFK members, Src, Fyn, Lck, Yes and Lyn are ubiquitously expressed in the central nervous system (CNS). These molecular transducers interact with, and participate in signaling from RTKs through different downstream pathways (MAPK, PI3-K, PKB/Akt, FAK) required to elicit neurotrophic responses such neurite outgrowth, myelination, axon guidance, proliferation and differentiation during CNS development [68–70]. In the developed CNS, STKs are involved in a number of additional functions, as diverse as regulation of neuronal apoptosis [71] or upregulation of ionotropic NMDAR (N-methyl-D-aspartate receptor) and other ion channels [72]. It is worth recalling that NMDARs are the main type of glutamate receptors that mediate fast excitatory transmission in central synapses, and are often located in lipid rafts. By modulating NMDARs, Src gates NMDAR-dependent synaptic potentiation and plasticity, critical for processes underlying learning and memory [72]. Aberrantly regulated STKs antagonize cell survival signaling pathways and

induce neuronal apoptosis. Excitotoxicity is a major cause of neuronal death in acute and chronic neurodegenerative diseases [73]. This phenomenon is initiated by overstimulation of glutamate receptors, leading to sustained intracellular calcium overload and the constitutive activation of calpains, a family of calcium-activated proteases [74]. Calpain-mediated truncation of Src triggers excitotoxic neuronal death by inactivation of downstream Akt survival signaling [75]. Further to their effects on Src, overactivated calpains also affect different kinases GSK3β and CDK5, which lead to hyperphosphorylation of tau protein [76], one neuropathological hallmark in Alzheimer's disease, as we will show in next sections.

Non-conventional trophic/survival factors involved in neuroprotection have been reported in lipid rafts, one of this factors being estrogen receptors (ER) [77]. The presence of ER in the plasma membrane of nerve cells was not without controversy because classical ERs (alpha and beta) are cytosolic proteins with no affinity for hydrophobic domains. However, it is now clear that a subpopulation of ERα is associated to the nerve cell membranes, and that they are responsible for non-conventional effects of estrogens [78]. The accepted model indicates that targeting of ERs to membranes may be achieved by palmitoylation [79] and this explains the presence of ERα in lipid rafts [80]. Recent evidence shows that activation of membrane ER trigger survival signaling pathways involving transient activation of Raf-1/MEK/MAPK cascade [78] in a synergistic crosstalk with c-Src-receptor tyrosine kinase pathways [81] are neuroprotective and prevent neuronal death in models of Alzheimer's disease [77, 78, 80].

Coherent interactions of functionally related proteins have been demonstrated in lipid rafts. The new dimension of complex physiological processes but biochemically related at a nanoscale level have led to the concept of signalosomes. A pioneering study by Chadwick and collaborators (2010) in brain cortical lipid rafts form a transgenic model of Alzheimer's disease (3xTgAD mice) have shown that synaptic and signaling networks are organized into multiprotein complexes in lipid rafts, enabling coherent clustering of synergistic signaling proteins. Remarkably, significant alterations in numerous receptor/cell signaling protein associations were detected in the transgenic AD model [82]. These finding are quite relevant for the disease in humans, as synaptic dysfunction is one of the hallmarks of AD [3, 83, 84].

Similar signaling platforms operate in neuronal mechanisms involved in neuroprotection against different toxic insults (including amyloid β). The signalosome described by our group in lipid rafts from human frontal cortex is particularly relevant because its implications in neuronal survival and death. Our recent research indicates that lipid rafts are the site of formation of a complex set of interactions between survival/growth factors ERα (described above) and IGF-1R, scaffolding Cav-1, NMDA receptor regulator PrP^c (physiological role), and ion channels pl-VDAC (a plasmalemmal form of mitochondrial voltage gated anion channel VDAC1) and NMDAR. This ER-signalosome likely contains signal transducers such heterotrimeric G-protein and STK such Raf-1 involved in neuronal ERα signaling. Unlike in other signalosomes, in this case, proapoptotic protein (pl-VDAC) share a common cluster with survival factors [80, 85, 86]. pl-VDAC has been found as a resident protein of lipid rafts in hippocampal and septal cell lines, mouse hippocampus and frontal cortex, and human cognitive areas, such as frontal cortex, septum and hippocampus [85, 86], suggesting that location of VDAC in neuronal rafts may be a general phenomenon. Although the exact role of this mitochondrial channel in cell membrane lipid rafts is still under debate, pl-VDAC has been claimed to participate in the extrinsic apoptotic pathway [87]. The presence of pl-VDAC (and perhaps NMDAR) in lipid raft ER-signalosome suggest that they might be a critical site involved in neuronal fate decision, a fact that might be relevant in AD neuropathology, as discussed in the next sections.

The preferential location of different neurotransmitter receptors (NTR) and ion channels has been demonstrated steadily since the discovery of lipid rafts in nerve cells, and the number of candidates keeps growing and far from being definitive. An excellent review and overview of neurotransmitter receptors and transporters associated with lipid rafts in neurons and glial cells can be found in [3]. The range of NTR involved is all-encompassing and include ionotropic receptors, such AMPA-R (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor), NMDA-R, $GABA_AR$ (γ-aminobutyric acid receptors) or nAChR (nicotinic acetylcholine receptors), metabotropic receptors such mAChR (muscarinic acetylcholine receptors), 5-HT-R (serotonin receptors) or mGluR (glutamate receptors), neurotransmitter transporters such EAAT (excitatory amino acid transporters), and many GPCR and G-proteins such Gα and Gβγ (See [3] and references therein).

Often, these proteins are not stable raft-resident proteins but behave dynamically and may traffic into or out lipid rafts, undergoing stimuli-induced integration in lipid rafts, which allow them interacting with transducers and even effector proteins (i.e. GPCR, G-protein-coupled receptors) or multimer formation (i.e. ionotropic receptors) to trigger either increase or dampen signaling responses. Lipid rafts also participate in the formation of neurotransmitter receptor clusters (i.e. NMDAR and $GABA_AR$ in postsynaptic neurons) influencing synaptic function, and are sites for endocytosis and trafficking of NTR. During neurotransmitter signaling, many GPCRs undergo agonist-induced endocytosis, leading to receptor recycling, sequestration and downregulation through clathrin-independent mechanisms [88, 89].

A number of neurotransmitter-independent ion channels have been found either as proper residents or transiently associated to lipid rafts. These proteins are generally downstream effectors of neuronal signaling and include the large family of voltage-dependent (Kv) potassium channels, Ca^{2+}-activated K^+ channels, different subunits of voltage-dependent sodium (Na_V) and calcium channels (Ca_V), VDAC and ClC families of voltage-gated chloride channels, G-protein-gated or cyclic nucleotide-gated (CNG) ion channels, transient receptor potential (TRP) cation channels, and water (AQP) channels [3, 52, 85, 90–92].

Structurally, ion channels are diverse in their architecture and topology, but generally contain several transmembrane domains, which allow integration within lipid rafts mainly through hydrophobic lipid-protein interactions with acyl chains of surrounding lipids within the lipid bilayers, and also with phospholipid head groups at the level of intra- and extracellular aqueous interfaces, being these lipid interactions absolute requirements for proper channel gating.

Further, members of all major ion channel families have been demonstrated to be regulated by membrane cholesterol and to partition into cholesterol-rich membrane domains [93]. Stability of channel proteins in lipid rafts is ensured by hydrophobic interactions of transmembrane domains with cholesterol within the core of the bilayer. Cholesterol itself not only provide channel compartmentalization but also alters the kinetic properties and current–voltage dependence of many voltage-dependent channels, particularly in voltage-gated Na^+ and K^+ channels [93]. Targeting of ion channels is isoform-specific, as demonstrated for Kv channels Kv2.1, Kv1.4 and Kv1.3, which are present in distinct membrane compartments in hippocampal and cortical cells [90, 94]. Apparently, membrane domain cholesterol levels differentially modulate the trafficking and localization of Kv channels [90, 95]. Overall, ion channels targeting to lipid rafts channel induces not only clustering to other raftphilic partners, but also modulation of ion channel gating by virtue of microenvironmental cholesterol.

Finally, lipid rafts are associated with cytoskeleton [96]. Interactions with cytoskeletal components (actin, tubulin, vinculin, filamin, and tau) contribute

to the regulation of lipid rafts assembly and clustering. The accepted view is that this depends on raft lipids, raft scaffold proteins and submembrane actin network [96, 97]. It has been proposed an intuitive model ("picket-fence") whereby actin filaments anchored to cytofacial leaflet of lipid rafts regulate lateral diffusion of adjacent membrane lipids and proteins [97]. Anchoring of actin filaments with raftphilic proteins have been proposed to allow the transient clustering and coalescing of small rafts to form larger homo - and hetero -GPI-anchored oligomeric rafts, through raft-based lipid interactions that generate functional raft domains [98]. This spatiotemporal microdomain clustering depends upon cholesterol, sphingolipids, phosphoinositol lipids and the cortical actin meshwork, where actin filaments cross-linked by myosin motors promote energy-dependent lateral movements of GPI-anchored proteins [98]. Besides actin, tubulin is also associated to lipid rafts and co-precipitates with caveolin-1 in brain extracts. In fact some authors have suggested that tubulin itself might behave as a scaffolding protein in lipid rafts [3]. Recent studies have shown that lipid rafts lipids are important elements in the interaction membrane-cytoskeleton. Thus, It has been shown that phosphoinositide lipids such as PtdIns(4,5)P2 and PtdIns(3,4)P2, which accumulate in the inner leaflet of lipid rafts, bind actin and direct actin assembly into filaments, and that PtdIns (4,5)P2 serves as a tubulin anchor on the plasma membrane [3, 96]. It is known that microtubule dynamics participate in raft-associated neurotransmitter signaling [3]. Certain G proteins have been shown to promote the GTPase activity of tubulin and to affect microtubule arrangement. The association between tubulin and heterotrimeric G proteins has been demonstrated to potentiate adrenergic and cholinergic signaling neurotransmitters by directly activating their respective G proteins [3]. Further, cytoskeletal dynamics and its interaction with lipid rafts are demonstrated to be directly involved in processes such neuronal growth, axonal/dendritic guidance, axonal regeneration and dendritic spine formation in hippocampal neurons [3, 96].

4. Lipid rafts in established AD

4.1 AD neuropathology: linking in lipid rafts disturbances

Alzheimer's disease is the most common neurodegenerative disease, and has reached pandemic proportions in developed countries. AD is characterized by progressive memory loss, cognitive deficits and subsequent gradual but relentless dementia. In the majority of cases AD occurs late in life and without a known cause (referred to as "sporadic" or "late-onset" Alzheimer's disease, LOAD). Brains of individuals with AD exhibit massive loss of synapses and neurons, as well as extracellular senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs). The most severe neuropathological changes occur in the hippocampus, followed by the association cortices and subcortical structures [99, 100].

The major proteinaceous component of SPs is a 40–42 amino acid polypeptide amyloid-β derived by proteolytic cleavage from the transmembrane protein APP. According to the amyloid cascade hypothesis (first proposed by Hardy and Higgins, 1992) [101], in the amyloidogenic pathway, the β-secretase activity of BACE1 (β-site APP cleavage enzyme 1), generates the amino terminus of Aβ [102] while γ-secretase complex (made up of four proteins: presenilin, APH-1, PEN2 and nicastrin) [103] cleavage at the carboxy-terminus and determines its length (Aβ40 and Aβ42). Aβ40 is the most common species and Aβ42 the more fibrillogenic and prone to aggregates in SPs [104]. As discussed below, lipid rafts are the key membrane domain for this sequential cleavage of APP. Conversely, in the non-amyloidogenic pathway, the zinc metalloproteinase ADAM10 named α-secretase, cleaves APP

within the Aβ domain [105, 106] and thus precludes Aβ formation. Of note, action of α-secretase occurs predominantly in non-raft domains [105, 106]. A reciprocal relationship exists between non-amyloidogenic and amyloidogenic APP processing such that impaired ADAM10-mediated proteolysis of APP serves to enhance amyloidogenic processing thereby elevating levels of Aβ peptides in AD-afflicted brains [105]. These assertions are extremely important for AD onset and progression, since it suggests a dynamic intramembrane and interdomain competition between proteolitic activities of α- and β- secretases on APP, which largely determine the balance between amyloidogenic and non-amyloidogenic patwways. Further, increasing evidence indicates that ADAM10 may also affect AD pathology through potential mechanisms including reducing tau pathology, maintaining normal synaptic functions, and promoting homeostasis of neuronal networks [106].

Fibrillation of amyloid β peptides is a critical and complex process largely responsible for amyloid toxicity [107]. The available evidence favors a model in which the conversion of the normally soluble Aβ peptide into insoluble oligomeric, protofibrillar, and fibrillar toxic forms [104, 107].

NFTs are also a pathological hallmark of AD, though not exclusive. NFT are also present in other taupathies such frontotemporal dementia [108]. NFTs contain abnormally hyperphosphorylated forms of the microtubule-associated protein tau, which causes detachment of tau from microtubules and the formation of insoluble tau aggregates. This leads to the occurrence of paired helical filaments and NFTs present in cell bodies and apical dendrites as neurofibrillary tangles (NFTs), but also in distal dendrites as neuropil threads, or in abnormal neurites associated to SPs [109].

A number of studies demonstrate that AD-associated cognitive dysfunction is strongly correlated with the accumulation of amyloid-β and hyperphosphorylated tau, however, the precise relationship(s) between neurological and biochemical hallmarks of AD remains incompletely understood, particularly in Sporadic Alzheimer's disease. Likewise, potential causes for AD remain unknown, except for the familial form of the disease (familial AD, FAD), in which several genetic mutations on proteins involved in amyloid β production have been well-identified [84, 110]. In FAD, which accounts for less than 1% of the total AD cases, rare autosomal dominant mutations have been identified in three genes, namely APP, PSEN1 and PSEN2, the latter two being the most common mutations found in FAD [84, 110].

Regarding the relationship between alterations amyloid-β and tau in AD-afflicted brains, current models suggest that amyloid oligomerization and aggregation drives tau hyperphosphorylation and fibrillation. By itself this modified form of tau stimulates cell dysfunction and neurodegeneration in AD both downstream and independently of Aβ [84, 111].

4.2 Lipid rafts and amyloid processing

Numerous studies have shown that proteins involved in amyloidogenic processing pathway APP, BACE1 and the γ-secretase complex are transmembrane proteins associated to different extents to lipid rafts (see the excellent review by Hicks et al., 2012) [6]. APP is localized in raft and non-raft fractions, but predominates outside rafts, while the β- and γ-secretases are mainly located in rafts. Noteworthy, APP and secretases exist in two pools, raft and non-raft, but their relative residence fraction vary depending on cellular signals and physicochemical microenvironmental factors (i.e. lipid composition of bilayer, see below). Conversely, the α-secretase involved in non-amyloidogenic pathway is membrane- but not raft-associated [106].

Regulation of APP raft localization involves interaction between the C-terminus of APP and flotillin-1 [112]. Further, another factor promoting raft localization of

APP is cholesterol. APP specifically binds cholesterol though a direct interaction with the C-terminal domain C99 (also known as β-C-terminal fragment, β-CTF) [113]. Apparently, binding of cholesterol to C99 would favor the amyloidogenic pathway in cells by promoting localization of C99 in lipid rafts [113].

Regarding secretases, raft localization of β-secretase and interaction with raft-resident lipids is mediated by palmitoylation of BACE-1 [114]. It has been reported that when BACE-1 is targeted to lipid rafts via GPI-anchoring, upregulation of amyloidogenic APP processing occurs and production of Aβ is increased [115]. Subunits of the γ-secretase complex are enriched in lipid rafts by means of S-palmitoylation of nicastrin and Aph-1 [116, 117] but, interestingly, does not directly modulate γ-secretase processing of APP [117]. Further, the lipid raft scaffolding protein caveolin-1 influences the γ-secretase spatial distribution favoring its partitioning to lipid rafts but also enhances its secretase activity [118].

Another important lipid-raft associated protein which was shown to play an important role in APP processing is PrP^c. Indeed, PrP^c regulates APP processing by inhibiting β-secretase activity in the cell surface, and this effect requires the localization of PrPc to lipid rafts [6, 119]. This led to the hypothesis that PrPc might be a key protective protein against AD, and that PrPc downregulation might impede the negative control of BACE1 activity and accumulation of Aβ peptide. However, no decrease of PrPc content has been reported in AD brains, therefore it is suggested that decreased ability of PrPc to control BACE1 might be consequence of age- and disease-dependent disruption of lipid rafts, at least in the case of sporadic AD [6].

The relationship of amyloid peptides and membrane components is often reciprocal. Several examples illustrate this bidirectional relationship. First, PrPc has been shown to be a receptor for Aβ oligomers (even at nanomolar concentrations). Binding of Aβ oligomers to PrPc results in the blockage of hippocampal LTP and reduction of PrP affinity for the NMDAR (through a complex allosteric modulation of its glycine binding site). Once out of the control by PrP, this results in steady-state NMDAR currents and excitotoxicity [6, 120]. Together with BACE1 regulation by PrPc explained above, this provides an integrated toxicity mechanism explaining the interplay between BACE1, PrPc, NMDAR, Aβ species and hippocampal LTP, in the hippocampal degeneration and functional decline in AD.

A second example is brain cholesterol. Within nerve cells, the biggest reservoirs of cholesterol are found at the plasma membrane, myelin sheaths and in the endocytic recycling membranes. The majority of brain cholesterol is derived from de novo biosynthesis, rather than from plasma LDL [121]. Cholesterol can directly modulate amyloidogenic secretase activities leading to altered amyloid-β generation [10, 122–124]. Collectively, these data indicates that elevated cholesterol levels promote the co-clustering of APP and BACE1 in lipid raft domains, as well as their rapid endocytosis, and increases their activities. Conversely, experimental reduction of membrane cholesterol levels decreases the association of BACE1 with lipid rafts and reduces the activity of both BACE1 and γ-secretase, leading to additive reduction of amyloid-β production.

Cholesterol levels in the brain are regulated through a series of steps in a cross-talk between astrocytes and neurons (see excellent reviews by [125–127]). These involve HMG-CoA reductase (HMG-CoA, the rate-limiting enzyme responsible for cholesterol synthesis in neurons and glial cells), APOE-containing HDL-like particles released from astrocytes (which mediates the uptake of lipoprotein particles via LRP), LDL receptor-related protein (LRP, which serves as a neuronal receptor for astrocyte-produced APOE-containing lipid particles), ATP-binding cassette sub-family A member 1 (ABCA1, mediating cholesterol efflux from neurons has been also shown to modulate Aβ levels in neurons), and acyl CoA:cholesterol acyltransferase 1 (ACAT1, which converts free cholesterol into cholesteryl esters), amongst

other proteins. Excess free cholesterol in neurons is either converted to cholesteryl esters by ACAT1 or exported through ABCA1. Several lines of evidence indicate that cholesterol efflux, synthesis or esterification controls amyloid-β generation. Thus, stimulation of HMG-CoA or ACAT1 has been demonstrated to increase Aβ levels though mechanisms still poorly understood. Further, in vivo studies have shown that deletion of ABCA1 gene decreases the levels of APOE, a finding that correlates with greater amyloid-β deposits. Moreover, increased intracellular cholesterol (and perhaps cholesterol esters) has a considerable impact on membrane domain biogenesis and lipid raft formation, eventually leading to stimulation of amyloidogenic APP processing [128–130].

4.3 Untangling the conundrum of late-onset AD origin

Despite intense scientific research in the areas of genetics, molecular and cell biology, and neuroscientists throughout the world, causative factors for nerve cells destruction in LOAD are far from conclusive and have not been definitively established. Amongst factors evidencing solid links with neuronal loss and development of sporadic Alzheimer's disease are genetic polymorphisms, such ApoE4 [102, 103], neuroinflammation [104–106, 131], oxidative stress [107–110], neurolipid deregulation [111–114, 131], environmental factors, such chronic exposure to neurotoxic metals, pesticides or nanoparticles [115–117], dietary habits [118–121], and xenoendocrine and hormonal changes such menopause [1, 122, 123]. However, the only factor that is unequivocally associated to the onset of AD is aging. Aging is an extremely complex biological process affecting whole organism. Cerebral aging is acknowledged to involve multiple factors which converge to reduce cognitive functions such as mental speed, executive function, episodic memory, working memory, short-term recollection, spatial memory and capability to process new information, amongst other deficits [92, 124, 125]. These cognitive deficits are recognized to be secondary to losses in synaptic contacts, reduced neuroplasticity, dendritic branching, changes in neuronal and/or astrocyte physiology and crosstalk [126], and is accompanied by reductions in the volume of the hippocampus and pre-frontal, parietal, temporal and entorhinal cortical parenchyma [92]. Not surprisingly, brain areas which are more neuroplastic throughout life, such hippocampus and entorhinal cortex are most vulnerable to age and more prone to undergo pathological neurodegeneration [126]. Indeed, neurons that are particularly vulnerable in AD include the pyramidal layers of the hippocampus, those in layer II of the entorhinal cortex, and from certain areas of the neocortex (frontal, parietal and temporal cortices) [92]. Although most vulnerable neurons use glutamate as neurotransmitter (the most common in the brain), there is also significant loss cholinergic and noradrenergic neurotransmission in subcortical neurons in the basal forebrain [127]. In particular, the dysfunction of cholinergic neurons has received much attention (as per involved in obvious deficits in attention and memory in AD) and has been the stem for the "cholinergic therapy" in AD [127] Current knowledge support the notion that much of the cognitive dysfunction in AD is not due to loss of neurons containing a particular neurotransmitter, but to disruption of the network connections between key brain regions within the limbic system and specific areas of the neocortex [79].

My current view, shared with most neurologists and molecular and cellular neurobiologists, is that LOAD onset is determined by the slow but steady deleterious contribution of a combinatorial concert of factors referred above, superimposed to, and facilitated by both genetic predisposition and the exhaustion effect of lifestyle and aging. For instance, it is known that the apolipoprotein E allele e4 (*APOEε4*) expressed in the brain is a genetic risk factor for LOAD, whereas the e2 allele is protective. One copy of *APOEe4* increases the risk for AD by ~3-fold and two copies

by ~12-fold (http://www.alzgene.org), but its effect is magnified by aging, with a decrease in age at onset by ~5 years/e4 allele, in both sporadic and familial forms of AD [84, 132–134]. In line with this, the Society for Women's Health Research Interdisciplinary Network on AD, comprised of an expert panel of scientists and clinicians, has reviewed ongoing and published research related to sex and gender differences in AD, and defined the concert Age-APOE-Gender a triad of high risk for AD [133].

4.4 Lipid rafts: beyond and before amyloid possessing

The involvement of lipid rafts in AD extends well beyond facilitating amyloidogenic processing of APP or tau hyperphosphorylation. As described above, numerous neurotransmitter receptors, neurotrophic factors receptors and downstream signaling proteins, signalosomes, membrane trafficking components, ion channels and pathway effectors have been demonstrated to be differentially altered in Alzheimer's disease. Indeed, the number of cellular and molecular biological processes known to be presumably affected in AD is enormous. It is conceivable that not all these evidences occur in real degenerating human brains, as most observations have been obtained under artificial in vitro conditions, or in vivo using cellular and animal models, often overexpressing human proteins not normally expressed in experimental animals. These same arguments may also explain why contradictory results or fundamental controversies from different research groups are found in the literature. Furthermore, very relevant information from studies aimed at disentangling the pathological mechanisms for AD has been obtained from transgenic mice models expressing human components of the amyloidogenic pathway from well-established mutations in familial Alzheimer's disease. Thus, even if overexpressing transgenic models may render a disease scenario to closely resemble human amyloid and/or tau pathology, results are not necessarily translatable to the most common form of AD, i.e. LOAD. One plausible hypothesis which may assemble much information on the different mechanisms reported as altered in Alzheimer's disease is that they may belong to a programme of sequential set of events triggered at the onset of the disease, in some kind of self-destructive parallel domino effects, which are exacerbated during the progression of the disease.

In this sense, plentiful and compelling evidence point to lipid rafts alterations as a common underlying factor related to AD neurodegeneration, even at very early stages of the disease. Moreover, it is now clear that these structures undergo aging-associated modifications in brain areas even in subjects without signs of the disease. Overall, this suggests that it might be disentangling of lipid rafts a very early event in the transition from normal aging to developing this neurodegenerative disease.

It may be assumed that altered function of biochemical components integrated within lipid rafts may be secondary to destabilization of membrane structure of lipid raft, in particular with neurolipids. Indeed, a considerable number of studies demonstrate that lipid biochemical and biophysical anomalies lead to abnormal functioning of lipid rafts [10, 135–137]. These issues are discussed in the next section.

4.5 Lipid abnormalities and lipid rafts dyshomeostasis in AD

In the seminal description of the degenerative disease in 1932 named after him, Alois Alzheimer highlighted the occurrence of 'adipose inclusions' or 'lipoid granules' as the third pathological hallmark of AD. This finding did not receive enough attention until recently. Subsequently, biochemical alterations of lipid composition have been reported in post-mortem brains from individuals with AD. Perhaps, the intimate link between lipid metabolism and AD was only boosted when the ε4 allele of the

APOE gene was identified as a strongest genetic risk factor for LOAD [130, 134, 138]. The involvement of lipids in AD is substantiated by a number of epidemiological studies which support a role for cholesterol and essential fatty acids in the pathogenesis of AD [138, 139]. It is now well established that most, if not all, classes of lipids are implicated in AD pathogenesis. (recently reviewed in Chew et al., 2020) [140].

A wealth of studies have consistently demonstrated the depletion of LCFUFA in brain tissue from postmortem AD brains, in particular for fatty acids of the n-3 series, mainly docosahexaenoic acid (DHA) [131–144]. As mentioned before, brain is the organ containing the largest amount of DHA in the whole organism, and its depletion, underlie many alterations occurring during AD neurodegeneration. Indeed, DHA is a pleiotropic molecule. It is an essential component of nerve cells membranes associated to glycerophospholipids (mainly phosphatidylethanolamine, the most abundant phospholipid in nerve cells), and is largely determinant of physicochemical and biophysical properties of plasma membrane, such membrane viscosity, lateral mobility, phase separation and microdomain segregation, conformational transitions and lipid-protein and protein–protein interactions [60, 145, 146]. Besides, DHA is an active modulator of neurogenesis, synaptogenesis and neurite outgrowth and in memory consolidation processes [147, 148], but also in the activation of survival signaling pathways against oxidative and proinflammatory insults, amyloid β production [149–151], and transcriptional activation of neuronal antioxidant systems [152, 153]. The importance of DHA for brain health is highlighted by the extensive epidemiological and experimental evidence linking its depletion with the development of neurodegenerative diseases [154, 155].

Another evidence linking LCPUFA and AD is that LCPFA, especially DHA and AA are highly susceptible for oxidative stress. The high metabolic rate and elevated oxygen consumption in brain tissue, together with the enrichment in redox transition metals, such iron and copper, favor the free radical-induced peroxidation of LCPUFA in the brain parenchyma [156–158], and generation of reactive lipo- / endo-peroxides such isoprostanes, neuroprotanes, malondialdehyde, acrolein, and reactive aldehydes such HHE and HNE [159, 160]. Further, unlike other forms of free radical injury, lipid peroxidation is self-propagating and generated lipoperoxides react with membrane LCPUFA to produce additional reactive lipo-endoperoxides, to provoke extensive brain tissue damage [157, 159]. Obviously, one main outcome of lipid peroxidation is the structural damage of membranes, which impairs nerve cell physiology and finally causes cell death.

Pioneering studies published by our group on lipid rafts from human frontal cortex have demonstrated altered lipid profiles in AD brains at advanced stages V-VI, compared to control brains [161]. Amongst other alterations, lipid rafts displayed abnormally low levels of n-3 long chain polyunsaturated fatty acids (LCPUFA) and unsaturation and peroxidability indexes. LCPUFA, mainly docosahexaenoic acid (DHA; 22:6n-3), are particularly enriched in nerve cell phospholipids, and their presence is an absolute requirement for neuronal membrane function [125, 146, 162]. The results in this study were relevant for two main reasons. First, lipid rafts showed that, even in non-AD subjects, neuronal lipid rafts contain significant amounts of polyunsaturations in the form of n-3 and n-6 acyl chains, which makes them less packed and ordered than supposed. These findings are not surprising as fatty acids have the capacity to influence plasma membrane organization to facilitate intermolecular mobility (in a ´crowed´ protein environment such neuronal lipid rafts) by modulating membrane lipid composition, which affects functionality of lipid raft domains [145, 162]. Second, no changes in cholesterol were associated to lipid rafts in advanced stages of AD, which apparently contradicted the observation that AD brains contained higher cholesterol levels than normal brains. However, these observations are reconcilable on the basis that bulk

brain cholesterol may increase by affecting non-raft domains, without change in lipid rafts. In this case, interaction of rigid sterol ring of cholesterol with membrane phospholipids renders non-raft domains less fluid than normal, a notion which is supported by biophysical observations [59, 60, 128].

Other important rafts-associated lipids are gangliosides. These glycerospingolipids have been demonstrated to play a role as assembly- and aggregation-promoting factors [11, 17]. Aberrant levels and significant regional differences in the distribution of specific gangliosides have been observed in AD brains [10, 149]. Gangliosides are primary modulators of amyloid-β aggregation in AD, and it has been demonstrated that binding of GM1 to amyloid-β trigger conformational changes towards more ordered structures with increased β-sheet content, which correlates with higher toxicity [17, 163, 164]. A number of studies have revealed that gangliosides accumulate in senile plaques favoring the conversion of Aβ to a neurotoxic oligomers, and accelerates the formation of amyloid fibrils [152, 165, 166], these effects being favored in the presence of the ApoE4 genotype [167]. It has been demonstrated that Aβ has a high affinity for GM1 containing membranes both in vitro and in vivo, and that the N-terminal region of Aβ promote interactions with GM1 clusters in lipid rafts through hydrogen bonding and electrostatic interactions [13, 168, 169]. Further, the participation of gangliosides in the development of Alzheimer's disease is further strengthened by that fact that GM1 content in neuronal membranes, particularly in raft microdomains, increases with age [6, 152, 170]. In this sense, lipid raft GM1 acts as a 'seed' for amyloid-β aggregation [10, 151].

5. Lipid rafts alterations at early stages of AD

The presence of biochemical and physicochemical alterations in lipid rafts at early stages of AD has been recently reported [60, 171]. It is noticeable that lipid rafts are profoundly altered in the cortex of AD brains from the earliest stages namely AD I/II [172]. These changes affects the lipid matrix of lipid rafts well before the overt of clinical signs, and are retained as the disease progresses towards more advanced stages (stages III-IV) with little modifications. The most dramatic changes observed were the reductions in polyunsaturated arachidonic and docosahexaenoic acids, cholesterol, sphingomyelin, monounsaturated oleic acid, as well as increased levels of phosphatidylcholine and sterol esters [152]. Other reports have also shown elevated ceramide levels are and reduced sulfatides at the earliest clinically recognizable stage of AD [173], likely involved in oxidative stress-induced neuronal death.

Paralleling these changes, lipid rafts from AD frontal cortex displayed abnormally low unsaturation and peroxidability indexes, suggesting a high impact of lipid changes in physicochemical conditions of lipid rafts [60]. Lipid abnormalities in lipid rafts likely have a profound impact on membrane physicochemical properties, in particular to membrane order and microviscosity. We have shown that the reduction in n-3 polyunsaturated and the increase in saturated fatty acids, results in augmented density of hydrophobic interactions between saturated hydrocarbon acyl chains of phospholipids and sphingolipids within the membrane plane [11, 54, 55]. The consequences are: laterally condensed and more packed membranes, and higher physical order and microviscosity, in spite of the reduction in cholesterol [55]. These findings are in agreement with the observations in lipid rafts from the neocortex of aged APP/PS1 mice reported recently [54], which display a similar increase in membrane microviscosity secondary to reduced n-3 LCPUFA and cholesterol levels, as determined by steady-state fluorescence anisotropy [59]. Moreover, we have demonstrated that this transition towards more ordered membranes occurs during the initial stages of the pathology, and that it is correlated to the alterations observed in

the lipid profiles. A finding that is retained in intermediate stages of AD. The impact of these biophysical observations are likely relevant on the dynamics of amyloid aggregation. Indeed, it is known that interaction of Aβ with neural membranes is energetically more favorable in liquid-ordered membranes than in liquid-disordered counterparts and also that this association accelerate fibrillation [119, 158, 159, 174]. The relationship between liquid ordered-membranes and amyloid peptide association is reciprocal. Indeed, studies performed in rat synaptic membranes and in human brain tissue have shown that different Aβ peptides reduce membrane fluidity by partitioning into the hydrophobic core of membranes [119, 158, 159] thus adding additional membrane order to lipid rafts.

One relevant consequence of altered physicochemical properties of lipid raft observed in human brain cortex is that these likely modify interactions between raft resident proteins, in particular those involved in the differential processing of APP (see below).

Surprisingly, anomalies in lipid rafts from early AD stages are clearly more severe than those found in late stages (V/VI) [147]. It can be speculated that the neuronal metabolic collapse and/or disruption of neuronal lipid homeostasis [175, 176] in late stages of the disease, overcome membrane biosynthetic mechanisms to maintain lipid raft structure. In turn, this would weaken the thermodynamically unfavorable boundaries and tension line between raft and non-raft domains, eventually leading to more homogeneous membranes [11, 22, 24, 28].

Noticeably, we observed that lipid rafts alterations specifically affect frontal and entorhinal cortices in the same subjects, two brain areas particularly affected in AD, while no substantial effects are observed in the cerebellum. Further, Noteworthy, alteration in neurolipid levels and biophysical properties occurs in the frontal cortex at stages I/II, a brain region that devoid of neuropathological hallmarks of AD (neurofibrillary tangles and senile plaques) at such early phase [156, 157]. Moreover, It is worth mentioning that, at least in the frontal cortex, no astroglial proliferation is present at stages I/II, and very little at stages III/IV and mainly associated to senile plaques [156, 157]. Therefore, changes in lipid composition in lipid rafts in frontal cortex at early stages of AD pathology reflect modifications in the lipid composition of lipid rafts in neurons and cannot be explained by modifications in the neuron/astroglial ratio.

We extended our lipid analyses in the frontal cortex to entorhinal cortex and cerebellum, two other brain areas differentially affected in AD [109, 177, 178]. The results showed that alterations in lipid raft found in cortex are also present, and to a similar extent and disease-course, in entorhinal cortex [152]. It is known that enthorinal cortex is one of the first brain areas affected in AD, which exhibits the neuropathological traits at stages I/II [156, 157]. Overall, the fact that frontal cortex lipid rafts exhibit altered lipid profiles at stages AD I/II but not AD neuropathological hallmarks indicates that lipid raft destabilization develops well before the appearance of neurofibrillary tangles [100, 156, 157].

We have further explored the pathophysiological consequences of these alterations in the amyloidogenic pathway during development and progression of AD. As expected, we have detected main components involved in amyloidogenic pathway, namely APP, β-secretase and γ-secretase in lipid rafts from the three brain areas, in control and AD brains. We have observed that while the stage of the disease does not alter the level of association between APP-BACE and APP-PSEN1 in cerebellum, in the entorhinal and frontal cortices, the association between APP and BACE was considerably augmented when compared to the same areas in control lipid rafts. Conversely, physical association of APP and PSEN remained nearly constant between brain areas irrespective of disease stage. These findings are particularly relevant since β-cleavage of APP by BACE1 is the rate-limiting obligatory event, in the amyloidogenic pathway [6, 179, 180]. From a holistic perspective, the convergence

of APP and BACE to lipid rafts, allows a closer interaction between the two proteins facilitating β-cleavage of AβPP and eventually Aβ production [6, 179–182]. These observations point to the existence of homeostatic mechanisms precluding their unabated convergence under non-pathological conditions. In agreement, in a recent study in cultured hippocampal neurons, specific trafficking strategies that limit APP/BACE-1 proximity in has been demonstrated under physiologic states [162], therefore limiting amyloidogenesis. However, in this later study, disturbing raft architecture by moderate (but not severe) reduction of cholesterol levels increase Aβ production by enhancing BACE1 and APP interaction [161, 162, 164]. Our results in human brain lipid rafts, agrees with this finding that moderate reduction in cholesterol facilitates convergence pathways that routes APP and BACE to lipid rafts. However, the most important factors in triggering this convergence are the reduction in LCPUFA and the increased proportions of saturates/n3 and phospholipids/cholesterol in lipid rafts from entorhinal and frontal cortices, which, as we have showed before, gives rise to more liquid-ordered microdomains, likely stabilizing the interaction of AβPP and BACE1. In this sense, lipids can build a physical boundary between domains, circumscribing the β-secretase-APP complex within the lipid raft domain, where the pool of γ-secretase resides, thus favoring the sequential amyloidogenic cleavage of APP [183].

On the other hand, plasmalogens, membrane glycerophospholipids abundant neuronal lipids, have also been associated to AD. Reduced levels of these brain-specific lipids have been reported in AD brains [12, 184]. This is relevant for three main reasons: first plasmalogens (particularly plasmenyl-ethanolamine, PlsEtn) act as neuronal depots for essential LCPUFA in the brain and structural determinants of acyl chains packing and membrane order [23]; Second because the oxidative products of plasmalogens are unable to further propagate lipid peroxidation, and essential factor in triggering AD, thus plasmalogens may terminate lipid oxidation [185] and third, because they might have direct effect on the production of Aβ by inhibiting activity of γ-secretase [184].

Of particular interest is the fact that the normal aging brain undergoes a set of lipid alterations in lipid rafts collectively termed "lipid raft aging" [53, 94, 135, 151, 152, 168]. Changes affect levels of sphingomyelin, sulfatides and cerebrosides, LCPUFA, plasmalogens, phosphatidylinositol, gangliosides, and total neutral lipids (mainly cholesterol and sterol esters). Further, relevant relationships between main fatty acids and/or lipid classes detected in younger subjects, either disappeared or they occurred in the opposite direction [157]. Noticeably, these changes are mostly subtle but follow the same trend observed in early stages of AD. "Lipid raft aging" also involves changes in unsaturation and peroxidability indexes though they are significantly less severe than those reported in AD cortex [56, 57], and do not cause significant biophysical alterations of raft membranes. The significant reduction in peroxidability indexes observed in early stages of AD (reflecting the important reduction of LCPUFA in both raft and non-raft domains), and especially during lipid raft aging, is strongly indicative that oxidative stress and exhaustion of antioxidant systems are an essential part of AD neurodegeneration.

Interestingly, "lipid raft aging" exhibits clear gender differences and appear to be more pronounced in women, especially in older postmenopausal women [168], which strengthens a role for ovarian hormones in AD development. Indeed, according to the Alzheimer's Association [186] women have 2-fold greater lifetime risk of developing AD. Though still incompletely understood, it seems clear that menopausal transition and decline in estrogen adversely affect brain metabolism [187, 188].

Overall, the evidence accumulated point to a complex cocktail of factors, either endogenous and/or environmental, affecting lipid raft physiology and stability as paramount events in trespassing the thin borderline that separates normal and pathological aging [158].

6. Conclusions

In summary, we may conclude that lipid rafts are the neurobiological locus for the wealth of alterations involved in the molecular pathophysiology of Alzheimer's disease. Severe changes in the lipid matrix of lipid rafts represent the seminal event in the pathogenesis of Alzheimer's disease. These early changes, that selectively affect cortical structures altered in AD, have a profound impact on physicochemical properties of lipid raft which serves a favorable environment for the abnormal neuronal physiology, especially for the interaction of secretases and APP to trigger the amyloidogenic processing of APP and amyloid burden. This review argues in favor of lipid rafts dyshomeostasis representing a foundational effect on the onset and progression of this devasting disease, and opens the possibility for new pharmacological approaches and therapeutic windows to halt the initiation of this neurodegenerative disease.

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