Cholesterol and lipid rafts in the biogenesis of amyloid-β protein and Alzheimer's disease

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Abstract

Cholesterol has been conjectured to be a modulator of the amyloid cascade, the mechanism which produces amyloid-β (Aβ) peptides implicated in the onset of Alzheimer's disease. We propose that cholesterol impacts the genesis of Aβ not through direct interaction with proteins in the bilayer, but indirectly through inducing the liquid ordered phase and accompanying liquid-liquid phase separations, which partition proteins in the amyloid cascade to different lipid domains and ultimately to different endocytotic pathways. We explore the full process of Aβ genesis in the context of liquid ordered phases induced by cholesterol, including protein partitioning to lipid domains, mechanisms of endocytosis experienced by lipid domains and secretases, and pH-controlled activation of amyloid precursor protein secretases in specific endocytotic environments. Outstanding questions on the essential role of cholesterol in the amyloid cascade are identified for future studies.

Contents

1. Alzheimer's disease, Amyloid-β protein, and the amyloid cascade

Plaques and neurofibrillary tangles in the brain tissue of Alzheimer's disease (AD) patients have been implicated in AD since initial observations in demented patients at the turn of the 20th century(86). These plaques were determined to be principally composed of Amyloid-β (Aβ) protein, derived from cleavage of the amyloid precursor protein (APP). In 1991, it was proposed that a biochemical cascade starting from APP and ultimately resulting in the formation of these Aβ plaques is responsible for the genesis of AD(200, 81, 80).

The amyloid cascade hypothesis was proposed when many key details leading to production of Aβ plaques were unknown. Subsequently, the principal protein domains, cellular compartments, and lipid domains involved in the production of Aβ have been identified. Aβ oligomers have since become widely accepted as the potential Aβ agent responsible for neurogedeneration through thousands of peer-reviewed investigations(159). Despite extensive evidence in its favor, this hypothesis has faced controversy due to contradictory results in clinical trials targeting Aβ and the existence of evidence for a number of other hypotheses. The most prominent of these hypotheses is the Tau Hypothesis, in which the hyperphosphorylation of the Tau protein that makes up the microtubules of axons causes the formation of disordered neurofibrillary tangles observed in AD patients(142). This disrupts axon structure and thus the connection of neurons, directly leading to neurodegeneration. Other hypotheses include the Inflammation Hypothesis(137, 63), the Oxidative Stress Hypothesis(37), the Aβ membrane carpeting hypothesis, the pore hypothesis, and the detergent hypothesis(244). These hypotheses, however, do not rule out the amyloid cascade hypothesis, but may explain upstream or downstream events in the disease's progression. For example, hyperphosphorylation of Tau has also been directly connected to Aβ oligomers, and it may be that the amyloid and Tau disease pathways are synergistic(25, 105).

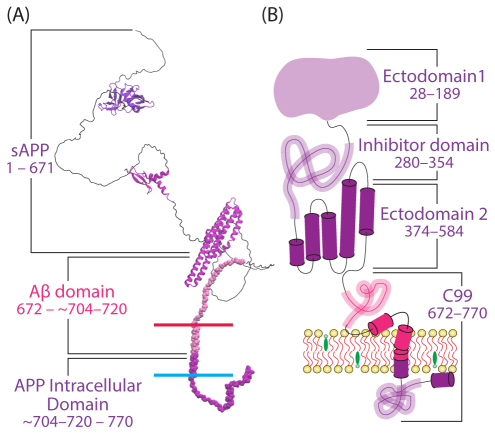

Figure 1

(A) All-atom representation of the amyloid precursor protein (APP) featuring structured domains predicted by Membranome (entry 117) based on AlphaFold2(56). Secreted APP (sAPP) domain produced from cleavage by β-secretase (BACE1), the variable amyloid beta domain (Aβ), and the intracellular domain visualized with bilayer ecto- and endo-plasmic domains indicated in pink and cyan. (B) Cartoon representation of APP highlighting structured ectodomains 1 and 2, the intrinsically disordered inhibitor domain, and the C99 peptide domain. Within the C99 domain is the variable Aβ subdomain, pending γ-secretase cleavage, as well as a transmembrane hinge at $G_{708}G_{709}$ evidenced to significantly modulate Aβ production.

Cholesterol has received significant attention as a promoter of Aβ formation. Proposed mechanisms for the action of cholesterol have largely focused on specific protein-cholesterol interactions that stand to impact Aβ-genesis or aggregation. However, at this time little is known regarding the physiochemical mode of action of cholesterol in the amyloid cascade. Cholesterol has been more generally implicated in the onset and progression of AD (especially late-onset AD). The broader discussion of cholesterol in AD falls beyond the scope of this review but has been reviewed by others in the past(71).

In this review, we present an overview of the lipid liquid ordered phase induced by cholesterol, lipid phase separation, protein partitioning to lipid domains, the principal proteins involved in the amyloid cascade, the endocytosis of these proteins to subcellular compartments, the modulation of function of these proteins by intracellular environment pH, and the current evidence for and against direct C99-cholesterol interactions. In this context, we provide evidence that cholesterol primarily acts as a modulator of Aβ generation not through direct interactions with APP and its secretases, but indirectly through solvation of the transmembrane helix and induction of the liquid ordered phase and the accompanying sorting of proteins to particular cellular compartments in which APP processing occurs.

1.1. The Amyloid Precursor Protein and the amyloid cascade

The protein signaling cascade that results in the production of Aβ begins with APP, canonically a 770-residue protein known to perform many functions including cell-cell signaling for synaptogenesis, regulation of copper levels, sphingomyelin and cholesterol homeostasis, and promotion of extracellular matrix development(154). Residues 1-671 of APP mostly

The role of Amyloid Precursor Protein in regulating cholesterol homeostasis

The role of cholesterol in the amyloid cascades should not be surprising given the evidence that APP is involved in regulating cholesterol biosynthesis and homeostasis. Pierrot et al. showed that moderate expression of APP in rat cortical neurons decreases cholesterol biosynthesis and negatively impacts cholesterol homeostasis, while the opposite effects were observed with significant decreases in APP expression (180). Recently, Mesa et al. further observed cellular defects during the differentiation and maturation of APP-knockout human induced pluripotent stem cells to human induced Neurons, but such defects were remedied through significant increases in cholesterol supply and membrane cholesterol content (146). Montesinos et al. propose that C99, the membrane-bound product of APP β-site cleavage by BACE1, acts as a lipid-sensing peptide for delivering cholesterol from the plasma membrane to the endoplasmic reticulum, forming detergent-resistant, lipid-raft-like, mitochondria-associated endoplasmic reticulum membrane domains (149). Normally, esterification of cholesterol in the endoplasmic reticulum enables the dissolution of these lipid rafts and cholesterol homeostasis; however, when C99 levels increase, lipid-raft formation is attenuated, resulting in an increase in BACE1 cleavage of APP (149). The discussion of how APP regulates cholesterol transport and homeostasis lies beyond the scope of this review, but it's important to recognize the significant cross-talk between cholesterol regulatory pathways and the amyloid cascade.

consist of intrinsically disordered domains separating three spectroscopically-resolved structured domains, ectodomain 1 (E1) in residues 28-189(45, 88), a serine protease inhibitor domain in residues 280-354(211), and ectodomain 2 (E2) in residues 374-584(46, 191), and a single-pass transmembrane (TM) domain in residues 699-724 (Figure 1)(41). The TM domain contains the sequence of Aβ_{42} in residues 672 to 713. APP homodimerization is shown to be primarily driven by E1-E1 domain association, dependent on both copper and heparin binding(45, 12), and the E1-E1 dimer conformation switches in response to pH(69). APP is canonically processed near the TM domain by two separate secretases, α- or β-secretase, each from the a disintegrin and metalloproteinase (ADAM) and beta-site APP cleaving enzyme (BACE) sheddase protein families and predominantly performed by ADAM10(120, 182) and BACE1(30, 259), respectively. While alternative APP processing can occur in ectodomain(7), endodomain(160), and Aβ domain(113) residues, the potential roles of these proteolysis reactions in AD genesis remain unclear. The endodomain of APP, residues 725-770, bind with several proteins such as G protein G0(76), adaptor proteins Fe65(195), X11(260), mDab1(172), and the kinase Jip1(198). Additionally, Y_{757}ENPTY_{762} are evidenced to be responsible for clathrin-mediated endocytosis. Aside from the role of clathrin binding in the endocytotic pathway of APP, which we review in section 4, the role of intracellular protein interactions involving the endodomain remain mysterious.

1.2. Enzymatic cleavage of APP and the biogenesis of Aβ

ADAM10 performs "α-cleavage" on APP at residue 687, producing secreted APP α (sAPPα) and the 83-residue TM protein C83 from the remaining 83 C-terminal fragment of APP. C83 does not contain residues 1-16 of Aβ, and thus cleavage of APP by ADAM10 ends the amyloid pathway. Instead, C83 proceeds along the complementary pathway (Figure 2.A)(115). BACE1 performs "β-cleavage" of APP at residue 672, producing secreted

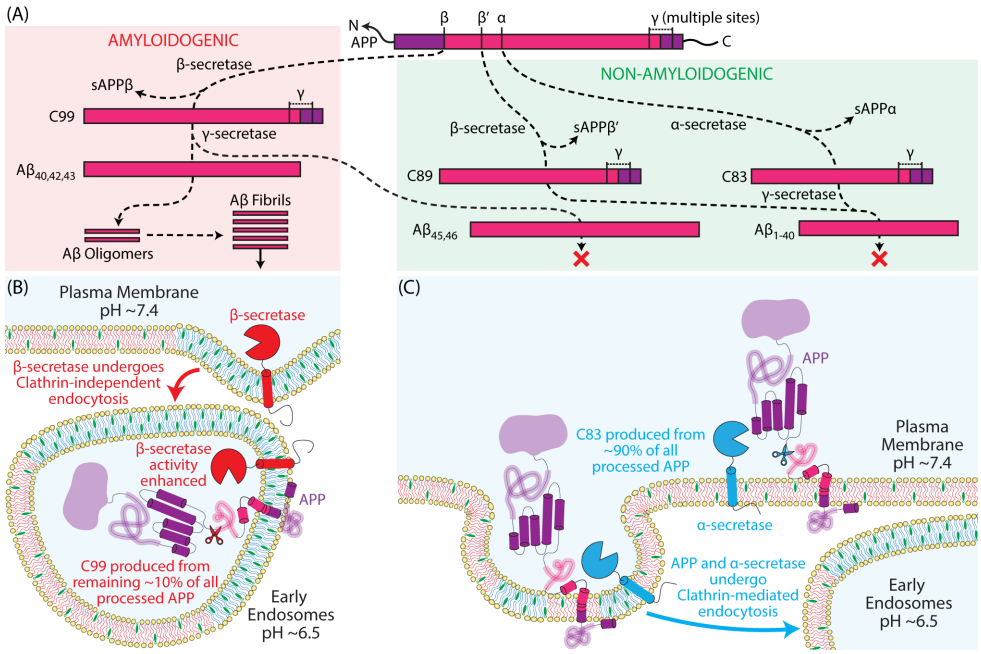

Figure 2

(A) Amyloidogenic and non-amyloidogenic APP processing pathways. (B) The first step in the biogenesis of Aβ is implied to occur in liquid ordered lipid raft domains (blue, saturated lipid tails) is the cleavage of APP by β-secretase (BACE1) to produce C99. This amyloidogenic cleavage of APP occurs primarily in early endosomes and accounts for 10% of APP processing. (C) Non-amyloidogenic cleavage of APP by α-secretase (ADAM10) accounts for 90% of all APP processing(240). It primarily occurs in the plasma membrane, implied to occur in liquid disordered domains (red, unsaturated lipid tails)

producing C83.

APP β (sAPPβ) and the 99-residue TM protein C99. C99 contains the Aβ sequence starting from residue 1, whose juxtamembrane and C-terminal helices have been resolved via NMR (Figure 2.B)(10, 98, 215). Alternatively, BACE1 can perform "β'-cleavage" of APP at residue 682, producing secreted APPβ' (sAPPβ') and the TM protein C89(53). Like with C83, C89 is processed by γ-secretase along the non-amyloidogenic pathway.

C99 is encapsulated by the multi-pass transmembrane protein complex γ-secretase, which performs a multi-step cleavage to produce Aβ(228). γ-secretase is composed of the transmembrane proteins presenilin 1 (PS1), presenilin enhancer 2 (PEN-2), anterior pharynx-defective 1A (APH-1A) transmembrane proteins. Additionally, it includes the single transmembrane helix and the ectodomain from nicastrin (NCT)(2). Residues D257 and D385 (TM helices 6 and 7) of PS1 catalyze the proteolysis of the C99 transmembrane domain (TMD) every 3- or 4-residues up the sequence until release of the product Aβ. This process starts at the membrane-cytoplasm interface at residue 48 or 49, which are the C-terminal end of the C99 TMD. Aβ ranging from 49 to 33 residues in length can be produced from this cleavage, although Aβ_{40} is the predominant product, at approximately 50%, in CHO cells(224). The produced Aβ can terminate the amyloid cascade through

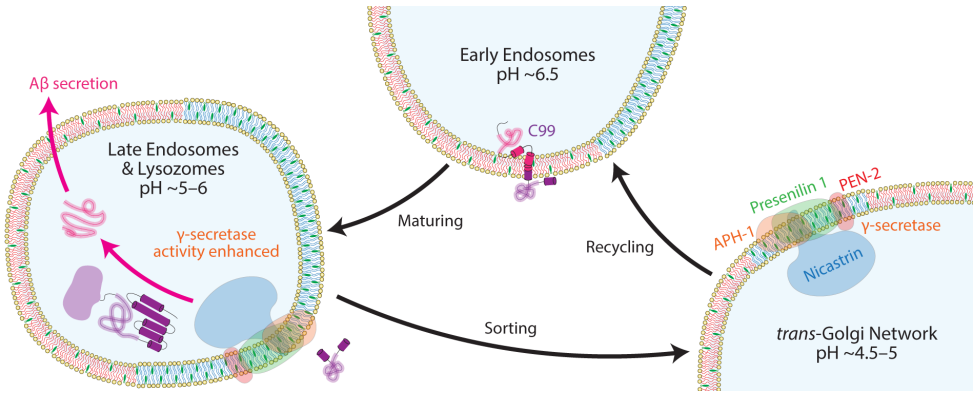

Figure 3

Processive cleavage of C99 by γ-secretase occurs in the trans-Golgi network, principally late endosomes, and results in the formation of Aβ. The cleavage process, which lacks fidelity, leads to the production of a distribution of Aβ isoforms, 33 to 49 residues in length, principally as Aβ_{40}(224).

the formation of fibrils and oligomers in either the intra- or extracellular space (Figure 3). A structure of cross-linked C83-γ-secretase complex was recently solved by cryo-EM spectroscopy, elucidating an anti-parallel β-sheet formed by a β-strand induced in APP residues V_{721}MLKK$_{725}$ (C99 residues V_{50}MLKK$_{54}$) interacting with two β-strands induced in the PS1 intracellular loop region connecting the C- and N-terminal domains of TM helices 6 and 7, suggesting that Aβ cleavage is performed at the helix-strand interface residues $T_{719}L_{720}V_{721}$ (C99 residues $T_{48}L_{49}V_{50}$)(263).

These proteins have multiple other known functions aside from proteolysis of APP. ADAM10 is known to proteolyze not only C99, but many other proteins(119), and mouse knockout experiments have shown that ADAM10 is required for cell viability(108). A notable example of critically-important proteins processed by ADAM10 is the Notch protein, which plays essential roles in signaling for cell differentiation, proliferation, and apoptosis(117). BACE1 is known to be particularly important for healthy axon myelination, potentially causing various mental disorders. However, BACE1 knockout mice remain viable and can reproduce(230, 132). γ-secretase is also known to be essential in many functions, acting as an enzyme for over 90 protein substrates, among which is Notch(252). Similarly to ADAM10, γ-secretase PS1 domain knockout mice are not viable(206). The many and diverse critically important functions of these proteins have made the development of drugs, aiming to prevent the progression of AD by modulating of Aβ production a challenging task(171).

Familial Alzheimer's disease (FAD) mutations, which cause early-onset AD (onset at under 65 years of age), occur in APP, PS1, and presinilin 2 (PS2), a homologue of PS1(15). Mutations in ADAM10 can attenuate APP processing and may contribute to late-onset AD(233). It is not yet known if there are BACE1 mutations that influence AD genesis and progression(227). Within APP, most FAD mutations appear in the C99 domain near the BACE1 cleavage site, the E1 copper binding site (important for copper reduction), the ADAM10 cleavage site, and the γ-secretase cleavage site(170, 22). Many FAD mutations also appear in the PS1 domain of γ-secretase. However, though these mutations

are distributed across various regions within PS1 such that it is difficult to elucidate the biophysical mechanism by which they modulate Aβ production(193).

Mutations to APP in the C99 domain can influence trafficking of APP to different subcellular compartments, ultimately impacting the propensities for α- or β-cleavage(129). The propensity of APP, ADAM10, BACE1, and γ-secretase for localization to subcellular compartments of distinct lipid domain composition and pH are of central importance to determining the products of these biochemical cascades.

2. Effect of the membrane on C99 structure and homodimer stability

The transmembrane domain of C99 features the sequence $G_{29}xxxG_{33}xxxG_{37}G_{38}xxxA_{42}$ which includes multiple Gxxx[G/A] glycine zipper motifs. Within the C99 GxxxG repeat motif lies a "GG hinge" at $G_{37}G_{38}$ in the TMD, initially identified by molecular dynamics (MD) simulations(148) and confirmed by NMR and EPR experiments(10, 155), which is conjectured to be important to processing by γ-secretase (Figure 1)(177). Hydrogen-deuterium exchange studies also observed side chain(177) and alpha helix(33) hydrogen bonds to be substantially weaker near the GG hinge, suggesting the amide bonds are readily available for γ-cleavage.

At the C-terminal end of the TMD, residues A42, T43, V44, I45, V46, T48, L52, and K53 all feature several mutations found in AD(250). Some mutations to these residues decrease the propensity for homodimerization(258), and enhance Aβ_{42} production(55). A "lysine anchor" formed by the triple repeat $K_{53}K_{54}K_{55}$ is evidenced to register at the C-terminal end of the TMD membrane surface(121). While these key sequences have been used in analyzing the structure of C99, much work remains to be done to identify the roles of specific residues in the initiation and termination of processive cleavage of C99 by γ-secretase and the resulting Aβ isoform distribution.

Identification of the GxxxG motif

This sequence motif appears in the transmembrane alpha helical domain of GlycophorinA (GpA), $I_{73}TLII_{77}FGVM_{81}AGVI_{85}GTIL_{89}LISY_{93}GI$, and has principally been used as a reductive model for understanding transmembrane helix-helix association since discovery the of its reversible homodimerization via SDS PAGE assays by Furthmayr and Marchesi(74). Engleman and coworkers found that subdomain dimerization characterized via SDS PAGE was significantly reduced by the substitution of residues L75, I76, G79, G83, V84, and T87(124). Subsequently, they found that a poly-Leu model maintaining only the LIxxGVxxGVxxT motif was sufficient for homodimerization(125). Conformational characterization of the GpA homodimer was elucidated for GpA in micelles(134) and bilayers(212), quantifying the presence of characteristic glycine "grooves" along the alpha helical face which provide specific sites for alpha helix dimerization and a right-handed superhelix(231). The GxxxG motif was later found to appear in abundance in membrane protein sequences(194, 201). The glycine alpha carbon was later found to stabilize the transmembrane structure by serving as a hydrogen bond donor(202, 153), and that alanine serves as a next-best substitution in the GxxxG motif(4). The GxxxG motif and the predominant right-handed superhelical structure stabilized by this motif have become a fundamental unit of transmembrane protein structural characterization and design(150, 106, 136).

2.1. Changes in membrane thickness, curvature, and phase affect APP C99 domain structure and dimerization

Thickening of the membrane reduces the relative amount of Aβ_{42} and Aβ_{43} produced while leading to an overall increase in γ-secretase activity(89, 251). Additionally, increasing the membrane curvature is found to increase the magnitude of fluctuation of the GG hinge and the overall tilt of the TMD(59). It is likely that the magnitude of fluctuations in the hinge may enhance Aβ_{42} and Aβ_{43} production(10).

It has further been noted that the membrane thickness can preferentially stabilize and environmentally select specific C99 dimer conformations(57, 58, 128, 246). Beyond the hinge lies another glycine zipper motif, $G_{38}xxxA_{42}$, often found in TM dimers(4), important for C99 homodimerization(8). The GxxxG repeat motif appears to facilitate C99 dimer formation in thicker membranes while the competing GxxxA motif supports dimers most often observed in thinner membranes and micelles(13).

2.2. Effect of membrane on C99 monomer, homodimer, and potential role in Aβ production

The competition between C99 homodimerization and C99 monomer cleavage by γ-secretase plays an important role in Aβ genesis. The single-pass transmembrane protein binding site in γ-secretase is evidenced to only accommodate monomeric proteins, such that C99 must be monomeric to be cleaved to produce Aβ(252). Song et al. used EPR experiments to determine the dissociation constant of the dimer in 3/1 16:0-18:1 PC/16:0-18:1 PG (3/1 POPC/POPG) vesicles to be 0.47 $\pm$ 0.15 mol% (-3.2 kcal/mol), a weak propensity for homodimerization(215). NMR spectra of C99 in micelles of the single-tail 14:0 Lyso PG (LMPG) and bicelles with 7:0 PC (DHPC) detergent performed at lipid/detergent ratios (q) below 1 have been found to be nearly indistinguishable(10, 13, 216). It may be that past NMR characterizations of full-length C99 in DHPC bicelles are not wholly representative of C99 structure in lipid bilayers.

Caldwell et al. characterized DHPC bicelles with $q < 1.0$ using small-angle X-ray and neutron scattering, fluorescence anisotropy, and MD simulation, finding $q \leq 0.5$ bicelles to be spheroidal (micellar) in shape, due to mixing of DHPC with other lipids(31). Additionally, Piai et al. demonstrated that reducing q below 0.7 manifests substantial changes to NMR spectra as q decreases(179). To address this, recent solution phase bicelle experiments have been performed using n-dodecyl-β-melibioside (DDMB) detergent to successfully solubilize C99 in a variety of lipid compositions including both 14:0 PC (DMPC):egg sphingomyelin (eSM):cholesterol (Chol) (4:2:1) and POPC bicelles(98). These DDMB bicelles were observed to achieve the ideal bicellar disk shape via SAXS and Cryo-EM.

DDMB bicelles were confirmed to solubilize a distribution primarily composed of C99 monomers, a significant population of C99 homodimers, and rarely-observed C99 homotrimers. A particularly notable result is that residues 3, 4, and 64-74 were observed to be mobile but inaccessible to both hydrophilic and hydrophobic paramagnetic probes. Overall, the results are suggestive of a C99 homodimer primarily stabilized by a complex ensemble of conformations in which extramembrane domains form transient secondary structures that can occlude paramagnetic probes from interactions with peptide backbones.

Following this work, we have recently published explicit solvent simulations of the full-length C99 homodimer and monomer in POPC sampled using generalized Replica Exchange with Solute Tempering MD(166). We observed that the extramembrane domains of the

homodimer, including the Aβ subdomain K_{16}LVFFAED$_{23}$ and the C-terminal juxtamembrane subdomain Y_{57}TSIH$_{61}$, form metastable inter-protein β-strands that are otherwise intrinsically disordered in the monomer. These homodimer-induced extramembrane secondary structure elements apparently cooperatively stabilize the homodimer GxxxG interface. Further experimental and simulation studies are required to fully understand the role of sequence and membrane environment, including phase separation into lipid domains, in defining the C99 monomer-dimer equilibrium.

2.3. Cholesterol interactions with Amyloid Precursor Protein

Cholesterol has been proposed to modulate C99 conformation through a C99-cholesterol complex. The C99-cholesterol complex was first suggested by Sanders and co-workers based on observations of shifts in the 1H-15N 2D NMR spectra of C99 in the TMD as a function of the concentration of cholesterol analogue β-CholBIMALT in LMPG micelles(13, 14). Subsequent experiments by Barrett et al. in DMPC:DHPC bicelles led to the proposal that C99 binds to cholesterol via the GxxxG motif in the TMD(10), the same glycine zipper that stabilizes C99 homodimers(148, 58, 101). It was further proposed that the binding site is completed by formation of the C99 juxtamembrane helix.

Song et al. developed a phenomenological kinetic superequilibrium constant describing competing dissociation constant between C99-C99 homodimer and C99-cholesterol complexes with which the C99-cholesterol dissociation constant was found to be 2.7 $\pm$ 0.3 mol% (-2.1 kcal/mol)(215). However, this C99-cholesterol dissociation constant is weaker than typical protein backbone-water hydrogen bonds(70). APP and the C99 substrate do not contain multiple transmembrane helices necessary to bind cholesterol, as found in various other transmembrane proteins(139). Because of this weak estimated C99-Chol association, extensive unbiased atomistic MD simulations have been used to investigate how cholesterol interacts with and modulates monomeric $C99_{16-55}$ structure at a variety of concentrations, juxtamembrane (JM) domain K_{16}LVFFAED$_{23}$ protonation states, and in the presence of FAD mutants in the JM domain(168). This recent simulation work suggests that cholesterol modulates C99 structure via ordering the surrounding lipid bilayer environment, and weakly interacts with C99 around the TMD with weak specificity, appearing to act as a co-solvent rather than as a ligand.

This result makes sense in the context of the shift in chemical shifts initially observed by Beel et al., from which cholesterol-responding residues were identified around all faces of the TMD domain(13). Cholesterol is also evidenced to enhance the interaction of Aβ with the membrane surface. Using atomic force microscopy (AFM), Gao et al. and Kandel et al. demonstrated that Aβ pores can be activated within cholesterol concentrations in which phase separation is observed in a ternary mixture, and deactivated when the cholesterol concentration increases to the point where phase separation disappears(75, 109). Fantini et al. demonstrated that Chol:ganglioside (GM1) binary mixtures and Chol:GM1:POPC ternary mixtures, which form the liquid ordered (L_o) phase and L_o-liquid disordered (L_d) phase separation (discussed in section 4.2), respectively, stabilize GM1 conformations which form strong hydrogen bond interactions with Aβ, facilitating Aβ aggregation on the membrane surface(68, 99). Rather than through specific protein interactions with cholesterol, cholesterol seems plays its role in controlling the amyloid cascade through ordering the lipid environment (Figure 4). It may be that the L_o phase is principally responsible for modification to the behavior and conformation of APP, C99, and Aβ that are often attributed to

specific interactions with cholesterol. Investigative work on the role of lipid phase on APP TMD and its secretases requires experiments or molecular simulation approaches that can reliably capture atomistic details of cholesterol and protein in lipid bilayers.

There are interesting parallels between the discussion of the possible roles of cholesterol in Aβ genesis and aggregation and that of the role of urea denaturant in protein folding. Competing theories were developed to account for the mechanism of action of urea in facilitating protein unfolding(192). The "direct action" theory posited that urea changed the nature of the aqueous solution, rendering it less polar and thereby relatively stabilizing the unfolded state of the protein(95, 247, 32). Conversely, the "indirect action" theory proposed that urea directly competes for hydrogen bonding with backbone amide and carbonyl groups, thereby stabilizing the unfolded state of the protein(118, 17, 48). It is our view that "direct" mechanism best explains urea's role in protein unfolding, while the "indirect" mechanism best describes the role of cholesterol in Aβ genesis.

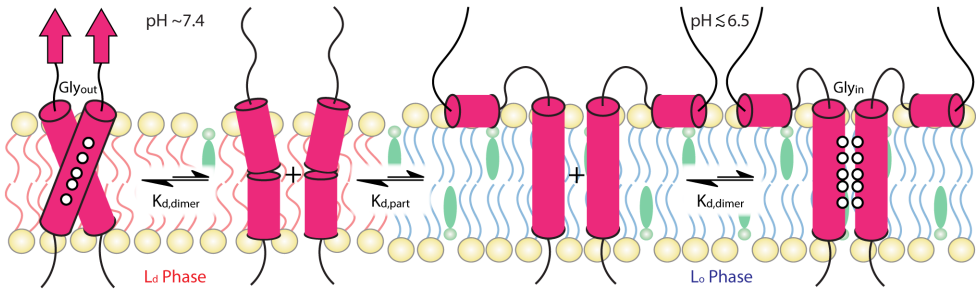

Figure 4

In bulk-like environments of liquid disordered phase (red) APP transmembrane domain is evidenced to form a relatively higher population of Gly-out homodimer(58) which can be stabilized with metastable extra-membrane β-strands(166). In the neutral pH plasma membrane, the JM domain K_{16}LVFFAED$_{23}$ α-helix is destabilized(165, 170). In raft-like environments of liquid ordered phase (blue) APP transmembrane domain is evidenced to form a relatively higher population of Gly-in homodimer, stabilizing the dimer with glycine zipper (white circles) C$\alpha\cdots$C=O hydrogen bonds. In the acidic endosomal membrane, the JM domain α-helix is stabilized(98, 155).

3. Role of cholesterol and membrane in the amyloid cascade

The role of cholesterol in modulating the amyloid cascade has been the subject of much speculation(36, 52). The onset of AD has long been correlated to enhanced levels of cholesterol resulting from diet, genetic predisposition, or aging(210, 189, 188, 207, 183, 266, 257, 253). Enhancements to cellular cholesterol concentration are also observed to up-regulate Aβ synthesis(188, 140). There are many non-membrane enzymes which specifically bind to cholesterol via a wide array of motifs. Most cholesterol-binding proteins perform oxidation, hydroxylation, sulfonation, or esterification of the head group(127).

In the membrane, cholesterol has been observed to complex with multi-pass transmembrane proteins. Most proteins found to complex with cholesterol are G-protein coupled receptors (GPCRs), which feature a cavity formed by the characteristic GPCR heptad of transmembrane helices(139).

3.1. Lipid rafts and protein partitioning to rafts is important to protein-protein interactions

The intracellular steps in the amyloid cascade involve processing of APP by its secretases in different cellular compartments: the plasma membrane (PM), early endosomes (EE), late endosomes (LE), and the *trans*-golgi network (TGN)(34, 138, 40). Mutations and post-translational modifications to these proteins are known to modify trafficking pathways and thus the ultimate production of Aβ. The first potential compartment in which APP may be processed is in the plasma membrane, in which ADAM10 can cleave APP to abort the process leading to the production of Aβ. The cellular compartments and trafficking pathways involved in Aβ production are described in detail in section 4.

Protein-protein interactions are not only dictated by subcellular compartment localization, but are also dictated by wether proteins laterally compartmentalize into dense, microscopic domains defined by concentrations of cholesterol, sphingomyelins, and gangliosides often called "lipid rafts"(209). Certain membrane proteins are evidenced to preferentially partition into these cholesterol-rich domains from the bulk membrane, driven by sequence and post-translational modifications(130). The existence and potential role of lipid rafts has been the subject of controversy. While L_o domains sharing properties characteristic of lipid rafts have been observed *in vitro*, raft domains have proven difficult to observe *in vivo* owing to their nanoscopic size and limited lifetime. Nevertheless, there is a developing consensus that lipid rafts persist in plasma membranes at the nanoscopic scale, identified using FRET, fluorescence quenching, super-resolution diffusion, electron microscopy, and single-molecule tracking(126).

3.2. Lipid phase is a sensitive function of cholesterol level and lipid tail saturation

There is a rich literature of *in vitro* lipid bilayer experiments, simulations, and theory that have elucidated the direct role played by cholesterol in inducing lipid raft formation through the formation of the L_o lipid bilayer phase from the L_d phase due to the co-localization and condensation of saturated lipids and sphingomyelins with cholesterol.

In binary saturated lipid:cholesterol mixtures, the main lipid phase transition temperature (T_m) from the gel (S_o) to L_d phase and bilayer to non-lamellar phase temperatures are

Identification of the liquid ordered phase

The condensing effect of cholesterol in lipid layers has been known for approximately 100 years, initially observed in lipid monolayers on aqueous films(122). Quantitative characterization of lipid structure and dynamics in solvated lipid bilayers has principally been achieved using residual quadripolar splitting of the deuterium NMR power spectrum, used to determine the carbon-deuterium order parameters, as detailed by Seelig(199). Brown and Seelig performed some of the first investigations of the effect of cholesterol on 16:0 PC (DPPC) phosphate group(28), followed by Smith and coworkers who quantified the ordering of slightly shorter saturated lipid DMPC acyl chains in the presence of cholesterol(61). The first binary phase diagram featuring the L_o to L_d phase transition was determined by Vist and Davis using the carbon-deuterium order parameter for binary mixtures of DPPC and cholesterol(245).

broadened until the L_o phase fully forms at about 20 mol% Chol, at which the S_o is apparently abolished. Detailed all-atom MD investigations have revealed that the L_o phase is not homogeneous, like L_d and S_o phases, but instead features a coexistence of L_d and nanoscopic S_o-like hexagonally-packed domains (Figure 5.A)(167, 169, 213, 102). The S_o phase can be abolished due to presence of cholesterol(87, 93, 100, 110, 147) or when saturated lipids are in mixtures with unsaturated lipids(131, 178, 221, 254), which lower the T_m of saturated lipids. In multi-component lipid bilayers, as the local concentration of cholesterol complexes with saturated lipids or sphingomyelin either through non-equilibrium equilibration or in a critical fluctuation, the local L_o phase domain is expected to be introduced as continuous phase transition from L_d or S_o phases, depending on the system temperature.

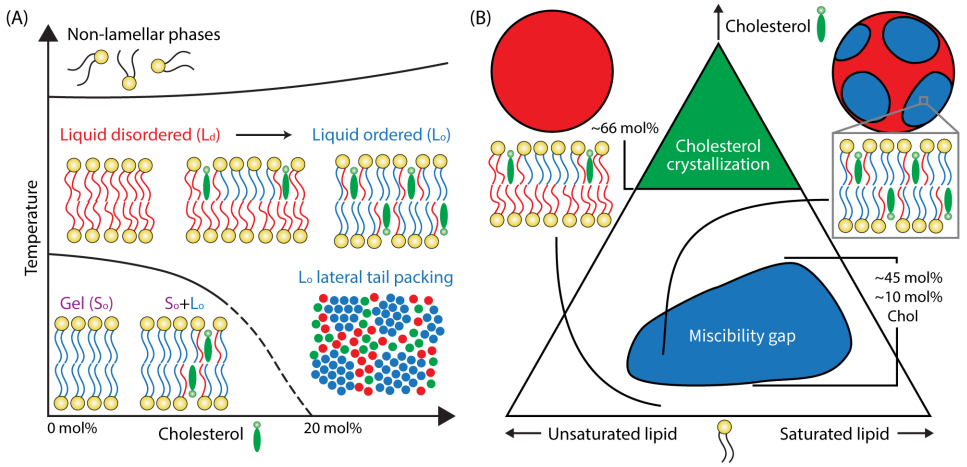

Figure 5

(A) Lipid phases and phase transitions exhibited for saturated lipid or sphingomyelin with increasing local concentrations of cholesterol. L_o lateral tail packing illustration is shown looking down the membrane normal. (B) Phase diagram representative of many mixtures involving saturated lipids or sphingomyelins, unsaturated lipids, and cholesterol at fixed temperature and concentration in aqueous solution in the lipid bilayer phase displaying the miscibility gap where phase separation is observed.

Over the past 20 years, many investigations have determined phase diagrams of ternary mixtures of cholesterol with lipids at physiological temperatures(141). Multiple points on phase diagrams of macroscopically-observable lipid bilayer phase separations resulting from mixtures of cholesterol, unsaturated lipids, and saturated lipid/sphingomyelins have been observed using fluorescence spectroscopy(69, 19, 24, 84, 91, 90, 103, 116, 162, 185, 219, 225, 232, 234, 236, 237, 238, 235), X-ray scattering(19, 38, 85, 176, 229, 261, 264), atomic force microscopy(24, 43, 111, 264), NMR(185, 238, 235, 50, 239), interferometric scattering(255), and Raman spectroscopy(5, 60), allowing us to achieve a general concept of ternary lipid mixture phase diagrams. Generally, at physiological temperatures ternary mixtures are observed to phase separate around equimolar, 1:1:1 molar concentrations of saturated lipid, unsaturated lipid, and cholesterol, defining a miscibility gap region inside the phase diagram in which systems are observed to form large, macroscopically-observed phase separations in the system (Figure 5.B). At low ($\lesssim$10 mol%) and high ($\gtrsim$45 mol%) cholesterol concentrations macroscopic phase separations disappear.

Above about 66 mol% Chol, cholesterol crystallizes and can crash out of the solution(100, 214), such that typical membranes can only accommodate up to 66 mol%(96, 173, 218, 249), though there are cellular membranes that can exceed this, such as the ocular cell plasma membranes(26). X-ray scattering experiments have revealed that ∼60 nm diameter domains of pure cholesterol domains can coexist with domains of saturated and unsaturated lipids at these high mol% cholesterol compositions(100, 264, 186, 265). Maze-like arrangements of cholesterol interlaced with lipid tails(169, 147, 173) may also form at high cholesterol concentrations due to propensity to form stable cholesterol homodimers(9, 64) and the unfavorability of cholesterol-lipid hydrogen bonding(47). A critical point, at which the system is unstable between phase separated and miscible states, appears at ∼40-50 mol% cholesterol, approximately equimolar saturated and unsaturated lipid concentration, and physiological temperature. Shaw et al. recently reviewed evidence for these critical points and their potential functional role (204).

3.3. Strong cholesterol affinity for saturated lipids and sphingomyelins

The strong preference of cholesterol to associate with saturated lipids and sphingomyelins over unsaturated lipids is another curious observation, and has led to speculations about the role of lipid rafts as platforms for facilitating specific protein-cholesterol(77) and protein-lipid(223) interactions. It is evidenced that there is a cholesterol L_o:L_d phase partitioning of approximately 4:1 and 2:1 in 20 and 40 mol% cholesterol based on experiments in phase separating DPPC:18:1 (Δ9-Cis) PC (DOPC) 1:1 mixtures including cholesterol. Supported monolayer Raman microscopy experiments performed by Donaldson and Aguiar determined L_o:L_d partitioning of cholesterol in 20, 33, and 50 mol% cholesterol at 3.9:1, 1.5:1, and 2.2:1, respectively(60). Similarly, Ma et al. determined cholesterol L_o:L_d partitioning to be 39.9, 3.63, 2.60, 2.17, and 2.13 at 10, 16, 20, 25, and 30 mol% cholesterol(133). The variable partitioning of cholesterol between L_o and L_d phases as a function of cholesterol concentration manifests due to the apparently high affinity of cholesterol for small domains of saturated lipids, and may allow for the formation of small L_o domains even with minority concentrations of cholesterol and saturated lipid in complex lipid bilayers. As such, even in conditions for which a macroscopic phase separation is not observed, there may yet be nanoscopic L_o domains which may play a role in various biochemical processes.

3.4. Phase separation and raft partitioning of proteins in the amyloid cascade

The structural and composition differences between the L_o phase of lipid rafts and the L_d phase of the lipid bulk play a role in modulating protein structure and function. APP, C99, ADAM10, BACE1, and γ-secretase are each suggested to preferentially partition into different lipid domains. The cleavage of APP and C99 is believed to typically occur in different cellular compartments and lipid domains for each complex. How and why these proteins prefer to localize and interact in such environments requires understanding the effect of these environments on protein structures.

Localization of proteins to membranes of the same subcellular compartment (discussed in section 4) is necessary but not sufficient for the association of two proteins to facilitate proteolysis. Within the same membrane, there can be a separation of lipids and proteins to lipid rafts which further compartmentalize the spaces in which protein-protein associations are likely to occur(217). There is general consensus in the literature that γ-secretase and BACE1 partition to lipid raft domains while APP and ADAM10 partition to the non-raft

membrane bulk, based on *in vivo* physiochemical analyses.

Increases in cholesterol concentration have been demonstrated to correlate with increases in Aβ production and enhancement of BACE1 in lipid raft domains(62). Fabelo et al. and Dìaz et al. analyzed the physiochemical properties of early AD human brain samples and found increases in lipid ordering and lipid raft formation caused by a relative decrease in unsaturated lipid concentrations. They also found a significant positive correlation of lipid viscosity with APP-BACE1 interactions(67, 54). Using healthy human and rat brain tissue samples, Hur et al. determined that γ-secretase is principally found in lipid rafts(97). Barros et al. used using atomic force to observe that γ-secretase preferentially partitions to L_o domains, and that γ-secretase can facilitate L_o domain growth(11).

Multiscale MD simulations of γ-secretase in a variety of membrane environments and cholesterol concentrations found that cholesterol can bind to the multi-pass transmembrane helices in a manner similar to that observed in GPCRs, and that the catalytic dyad of γ-secretase adopts a more active conformation in cholesterol-enriched bilayers(3).

Using endothelial cells, Reiss et al. demonstrated that an increase in unsaturated lipid concentration enhanced ADAM-mediated substrate cleavage, implying larger L_d domain bulk (reduction in L_o domains) functionally enhances ADAM activity(190). In recent work, we analyzed the effect of L_o and L_d domains on the structure of BACE1 and ADAM10 transmembrane domains in simulations. Our results revealed a definite difference in the character of the proteins in L_o compared to L_d domains(1).

The propensity of APP, ADAM10, BACE1, and γ-secretase to partition to L_o or L_d domains has been demonstrated to be a key determining factor in the kinetic processes leading to Aβ production. APP, BACE1, and γ-secretase also undergo palmitoyl post-translational modifications that can selectively enhance their enrichment in lipid raft domains, a phenomenon which has been quantitatively characterized via fluorescence experiments to affect the domain partitioning of proteins in general(130). We explore the role of palmitoylation in Aβ genesis in the following subsection.

3.5. Role of palmitoylation on protein partitioning and association

S-Palmitoylation is a reversible lipidation in which a palmitoyltransferase attaches palmitate to a cysteine residue via a thioester bond(104, 51). This post-translational modification can have numerous effects on a protein, including its subcellular localization and trafficking, its propensity to dimerize, and, particularly relevant to this review, its partitioning between L_o and L_d membrane domains(23). The role of palmitoylation in the amyloid cascades remains an open and active area of study.

Palmitoylation of APP C186 and C187 in E1 domains is observed to partition APP to lipid raft domains(20). These lipidation sites imply that the APP E1 domain inserts to the lipid bilayer in addition to the TMD, causing for a major conformational rearrangement of APP on the membrane surface. Changes to the structure, function, and protein-protein interactions attributed to an insertion of the E1 palmitoylation site to bilayers remain mysterious, but are correlated with the partitioning of palmitoylated APP into lipid raft domains(21) (Figure 6). Enhancing partitioning to raft domains and homodimerization may prevent APP processing by ADAM10 in the plasma membrane, enhancing the concentration of APP that comes to be processed by BACE1 in EE. Fluorescence spectroscopy experiments using cell-derived giant plasma membrane vesicles have demonstrated a strong preference for partitioning C99 to L_d domains in simple ternary phase separating membranes(35). This

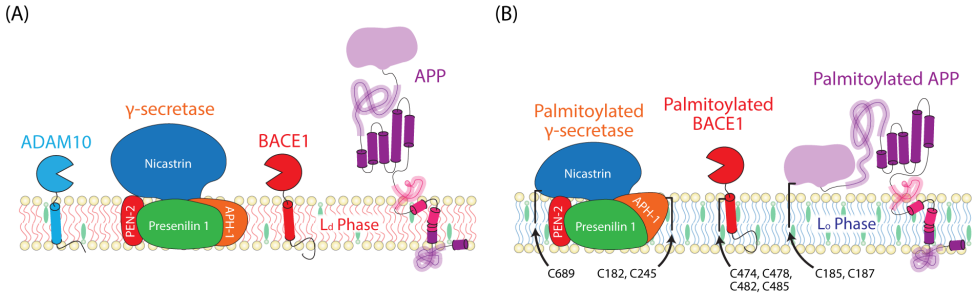

Figure 6

(A) Without palmitoylation, γ-secretase, BACE1, and APP may partition to L_d domains, along with ADAM10, which is not evidenced to be amenable to palmitoylation. (B) γ-secretase, BACE1, and APP are hypothesized to more likely partition to L_o domains upon palmitoylation (potential sites are labelled), which will cause conformational changes, particularly the association of extramembrane residues near the palmitoylated site with the lipid surface.

suggests an essential role for more complex lipid mixtures or C99-protein interactions in order to establish preferential partitioning of C99 to raft domains.

Palmitoylation of BACE1 may also play a role in Aβ production. BACE1 is known to be S-palmitoylated at residues C474, C478, C482, and C485 in and near its transmembrane domain(16, 243) (Figure 6). While experimental evidence agrees that the palmitoylation of BACE1 increases its affinity for lipid rafts, the effect of this on Aβ formation is still debated. Replacing the TMD and C-terminal domains of BACE1 with a glycosylphosphatidylinositol (GPI) anchor was initially found to increase Aβ and sAPPβ production(44). However, it has since been found that the GPI anchor leads to preferential BACE1 cleavage of APP at its β-site rather than its β'-site, resulting in an increase in amyloidogenic Aβ production without affecting the overall APP-cleavage activity of BACE1(241). Further, while raft-localization of palmitoylation-deficient cysteine to alanine BACE1 mutants is reduced compared to wildtype BACE1 in neuroblastoma cells, the mutations do not affect β-site cleavage of APP by BACE1 or amyloidogenic Aβ production(243, 152). More recent work exploring the same cysteine to alanine mutations *in vivo*, observed reduced raft localization of BACE1, cerebral amyloid burden, and cognitive decline in transgenic mouse models compared to wildtype(6).

The palmitoylation of other proteins involved, either directly or indirectly, with the amyloid cascades may be important for Aβ production. The S-palmitoylation of γ-secretase at C689 of nicastrin and residues C182 and C245 of APH-1 has been identified as an important factor for γ-secretase stability and raft localization(39). However, overexpression of non-palmitoylated nicastrin and APH-1 was not found to influence γ-secretase cleavage of C99 or C89(39). It has also been shown that palmitoylation of flotillins, which regulate the trafficking of APP and BACE1, is required for their membrane association(151, 157).

The interplay of palmitoylation of APP, the secretases, and other involved proteins is inherently related to the discussion of the role of membrane phases and cholesterol on Aβ production. Indeed, the aforementioned studies have revealed the critical role of palmitoylation on the proteins' partitioning; however, in many cases, the overall effect of palmitoylation on Aβ production is still debated. Further research is necessary to settle this controversy and understand how palmitoylation may be involved in AD development.

4. Role of cellular trafficking and pH in the amyloid cascade

APP, BACE1, ADAM10, and γ-secretase proteins mature from the endoplasmic reticulum (ER) to the Golgi apparatus and are transported to the plasma membrane (PM) via secretory vesicles along the constitutive secretory pathway(34, 138, 40). ADAM10 is particularly enriched in the PM(175), where 90% of APP cleavage occurs(240), resulting in secretion of sAPPα and production of C83(175). Endocytosis plays a critical role in the amyloidogenic pathway. APP and BACE1 are evidenced to be separated in the PM and encounter each other in EE(49) via clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE).

CME is a rapid endocytotic process which principally involves the formation of triskelia of clathrin protein that form a lattice, causing the membrane to bud and eventually pinch off part of the PM, forming and releasing a vesicle which develops into an EE(27, 83). ADAM10 and APP are both found to bind to the clathrin-adaptor protein AP2 and other adaptins which bind proteins in the membrane bulk for CME(259, 138, 161, 123, 29).

Phosphorylation of residues in the APP endodomain influences APP trafficking and amyloidogenesis. Among the phosphorylatable residues T729, S730, T743, and Y757(163, 203), phosphorylation of S730 enhances APP trafficking to the Golgi apparatus and Ala point mutation of T743 may enhance production of Aβ40 and Aβ42(72, 181, 197) and impacts APP interaction with some enzymes(222). Y757 phosphorylation has been identified to occur at higher propensity in AD patients and is suspected to prevent the interactions of APP with adaptor proteins due to its inclusion in the Y_{757}ENPTY_{762} motif(160, 181).

BACE1 is evidenced to be endocytosed through CIE, though AP2 is evidenced to be necessary for endocytotic recycling of BACE1(18). CIE occurs via binding with flotillin-1(82, 107), or ARF6(196). Flotillins are integral membrane proteins that support the formation of lipid rafts and facilitate endocytosis(164). ARF6 endocytosis requires lipid domains enriched in cholesterol, likely also forming lipid raft environments(156). As such, the partitioning of APP and ADAM10 to clathrin-containing bulk membrane phase and the BACE1 partitioning to environments with CIE-inducing proteins that are in lipid rafts is a likely explanation for why APP is processed by ADAM10 in the PM prior to endocytosis. Ultimately, changes in the lipid environment and pH are required for activating secretases in the amyloid cascade (Figure 7).

4.1. Endosomal environment pH activates BACE1 and γ-secretase, and discourages APP homodimerization

The role of pH in Aβ genesis and aggregation has received significant attention(114, 112, 256, 226). Unlike cytoplasmic and extracellular environments, endosomal compartments are acidic(174). pH lowers from $\sim$6.5 to 4 as endosomes develop from EE to LE to lysosomes(94). Both BACE1 and γ-secretase are active over a range of pH spanned by lysosomal environments and the PM. However, acidic subcellular environments are optimal for BACE1 and γ-secretase activity. BACE1 and γ-secretase are evidenced to be most active at pH 4.5(208, 78, 92, 65) and 6.5(145, 184, 79), respectively. BACE1 and APP have been visualized in HeLa cells and mouse neurons to be separate when trafficked to the PM(73), implying that BACE1 only processes APP upon associating together in acidic endosomal environments. Likewise, γ-secretase has been visualized in mouse neurons *in vivo* processing C99 in LE and lysosomes(135), and has been shown to be associated with lipid rafts in endosomes of mouse neuroblastoma(242).

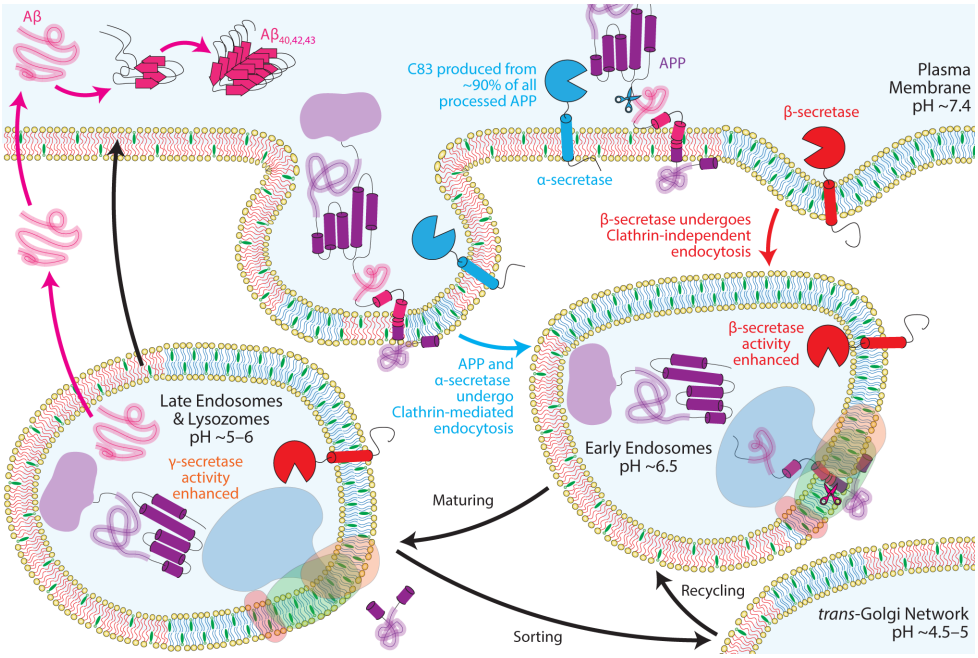

Figure 7

Illustration of subcellular compartments involved in the amyloid and complementary cascade pathways. APP, α-secretase (ADAM10), and β-secretase (BACE1) are represented in purple, blue, and red, respectively. Aβ is displayed in pink. The γ-secretase complex nicastrin, PEN-2, APH-1A, and presenilin 1 domains displayed in blue, pink, orange, and green, respectively. L_o domains represented with blue, ordered saturated lipid tails and a higher concentration of cholesterol, L_d domains represented with red, disordered unsaturated lipid tails and a lower concentration of cholesterol.

The majority of Aβ is evidenced to reside in the cytosol(262) and lysosomes where Aβ may oligomerize(66). In addition to controlling activity of BACE1 and γ-secretase, lower pH is observed to promote the formation of Aβ oligomers(144) via protonation of E22(112), which stabilizes the characteristic β-turn defined by hydrogen bonding between E22-K28. This β-turn may serve as a nucleus for Aβ aggregation(220). This observation suggests that pH shock could play a role in the nucleation and growth of Aβ aggregates by stabilizing aggregation prone N^* states exhibiting this critical β-turn(256, 143, 158, 187).

In low pH environments APP E1 domains are evidenced to undergo a conformational switch which may be important for modulating their homodimer dissociation constant and thus their availability for processing by ADAM10 and BACE1(88). Aside from ectodomains, residues with moderate pKa values and FAD mutants featuring charged residues are evidenced to play a significant role in determining the structure in the β-turn prone K_{16}LVFFAED$_{23}$ subsequence of C99 and Aβ.

Monomeric Aβ congeners including residues 1-28 have been demonstrated to form α-helices in very low pH environments upon a neutralizing mutation such as the E22Q FAD mutant using NMR(42). In C99 both low pH environments and E22Q D23N FAD mutants were observed in MD simulations to stabilize the juxtamembrane domain α-helix in C99

by neutralizing residues E22 and D23(168, 165). The stabilization of an α-helix in residues 22-28 precludes the formation of β-strands evidenced to be necessary to formation of stable Aβ oligomers and fibrils(159).

4.2. Role of pH and cholesterol-induced lipid phase on C99 structure and kinetics

This brings us to our attempt to understand how pH, FAD mutants to the JM domain, and cholesterol may make C99 available for processing by γ-secretase.

As discussed earlier, thickening lipid bilayers make the $G_{37}G_{38}$ hinge more rigid and reduce the quantity of Aβ produced by γ-cleavage(89, 251). However, γ-cleavage is believed to occur in thicker, liquid ordered domains. Barrett et al. suggested that cholesterol may specifically bind to C99(10), however Song et al. characterized the C99-Chol dissociation constant as a mere -2.1 kcal/mol, and unbiased MD simulations of their association demonstrated weak specificity for association of Chol around particular faces of the TMD (215).

Additionally, β- and γ-cleavage occur in low pH endosomal environments, where residues E22 and D23 in the C99 JM domain have been estimated to be protonated, thus stabilizing an α-helix in the JM domain(165), which seems to discourage formation of β-strands in the extramembrane domain and C$\alpha\cdots$O=C hydrogen bonds along the GxxxG zipper motif which stabilize the C99 homodimer(166). Moreover, formation of L_o domains by introduction of cholesterol into bilayers featuring saturated lipids has been demonstrated via MD simulation to promote α-helical structure in C99(168).

We believe the C99 homodimer is thus destabilized in L_o domains and at low pH, making C99 (potentially also full APP) within the endosomal environment more available for processing by γ- (and β-) secretase. It seems that in the L_d phase at neutral pH C99 homodimers are the most kinetically stable. But upon endocytosis to endosomal environments at lower pH and potentially higher concentrations of saturated lipids and cholesterol, C99 (APP) may be partitioned into lipid raft domains with γ (β) secretase, where the homodimer is less kinetically stable due to formation of the JM domain α-helix, and thus becomes available for processing (Figure 4).

5. Conclusions and future perspectives

In this Perspective, we explore several basic questions framed in order to better understand how lipid bilayers and cholesterol impact APP and Aβ genesis. The effect of varying cholesterol concentrations on lipid phase separations and the associated complex phase behavior is discussed. The effect of introducing the L_o phase via addition of cholesterol and the impact on the conformational ensemble of C99 is explored. The role of membrane structure, including thickness of the lipid bilayer, on the conformational ensemble of full-length C99 is discussed. Recent findings from NMR experiments and large-scale simulations regarding the nature of full-length C99 monomer and homodimer conformational ensembles are discussed. We summarize the known interplay of lipid phase separation, protein trafficking, and pH in the amyloid cascade.

In this perspective, we have noted competing theories for the role of cholesterol in Aβ genesis. The "direct action" theory posits that cholesterol establishes specific complexes with C99 that impact Aβ partitioning between lipid domains and cleavage by γ-secretase.

A competing theory of "indirect interaction," which has gained significant recent support, suggests that cholesterol impacts lipid phase separation and domain formation, which in turn impacts C99 structure, partitioning, and processing by secretases. In our view, the "indirect" mechanism best describes the cholesterol's mode of action in Aβ genesis.

Below we provide a summary of our current understanding and a list of future issues that we feel are well-suited for study at this time, using a variety of computational and experimental approaches described in this review.

SUMMARY POINTS

1. Observations of protein trafficking derived from *in vivo* studies of proteins in the amyloid cascade imply that APP and ADAM10 partition to L_d domains and BACE1 and γ-secretase partition to L_o domains. Direct observation using fluorescence probes has confirmed C99 strongly partitions L_d domains.
2. Cholesterol induces the formation of the L_o phase in bilayers upon achieving sufficient local concentrations in complex with saturated tail lipids. The modulation of lipid raft formation by cholesterol appears to be the principal, indirect mechanism by which cholesterol modulates the amyloid cascade.
3. Prior direct mechanisms for cholesterol modulation of protein structure via binding to membrane proteins, particularly binding to the single-pass transmembrane domain of APP (C99), might instead be attributed to the formation of the L_o environment.
4. Endocytotic trafficking is not only a mechanism by which proteins are sorted in the amyloid cascade. It is the principal determinant of when various stages of APP cleavage occur due to the pH activation of secretases and conformational changes in APP and C99.
5. Palmitoylation of APP, BACE1, and γ-secretase likely enhances the partitioning of these proteins to L_o domains. In addition, palmitoylation of BACE1 is conjectured to enhance production of Aβ.

FUTURE ISSUES

1. The partitioning coefficient of ADAM10, and BACE1 between L_d and L_o domains has not been quantified in any membrane system. Measurement of these partitioning coefficients would enable the development of kinetic models of Aβ production through which the role of L_o phase formation in Aβ formation may be explored.
2. Understanding the impact of pH, which differs between plasma membrane and endosomal environments, on the structure and function of APP, C99, ADAM10, BACE10, γ-secretase, and Aβ, and the encounter complex of all of these proteins, remains an important topic for future research.
3. Palmitoylation is an important factor in determining the domain partitioning of APP, BACE1, and γ-secretase. However, little is known regarding how palmitoylation impacts protein structure and partitioning between domains.
4. Cholesterol may modulate the structure, function, and cleavage of membrane proteins, beyond those discussed in this review, indirectly through domain formation

and protein partitioning. This may be particularly true for single-pass transmembrane proteins which lack cholesterol binding pockets, unlike the GPCRs for which direct cholesterol binding has been observed.

5. Lipid rafts have mostly been investigated as domains of cholesterol, saturated lipids, sphingomyelins, and gangliosides which facilitate protein-protein interactions. However, the role of protein concentration and partitioning coefficients in formation of L_o phase has received attention very recently(205, 248). Investigations of the effect of protein on L_o domain formation is of interest in general as well as in the specific cases of APP, C99, ADAM10, BACE1, and γ-secretase.
6. With the exception of CME facilitated by clathrin binding at the APP Y_{757}ENPTY$_{762}$ domain, the role of APP and C99 intracellular domain interactions with various cytosolic proteins in amyloidogenesis remains mysterious. Further characterization of the intracellular proteins that bind the APP intracellular domain is necessary to complete our understanding of the amyloidogenic pathway in relation to other biochemical processes.

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