Chapter 2

Mechanism of Alzheimer Amyloid β-Protein Precursor Localization to Membrane Lipid Rafts

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Additional information is available at the end of the chapter

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1. Introduction

Alzheimer's disease (AD) is a group of common neurodegenerative diseases associated with progressive dementia with aging. The principal pathological hallmarks of AD are senile plaques and neurofibrillary tangles in the brain, which are found at significantly higher frequencies in AD patients than age-matched healthy (non-AD) subjects [1]. Senile plaques consist mainly of 39–43 amino-acid amyloid-β (Aβ) peptide, which is generated by sequential proteolytic processing of amyloid β-protein precursor (APP) (Figure 1) [2]. Common Aβ species generated in the human and murine brain are Aβ40 and Aβ42. Mutations in *APP* and *Presenilin*, which have been identified as familial AD-causative genes, result in increased Aβ production and/or an increased ratio of neurotoxic Aβ42.

Aβ is generated by sequential processing of APP with β- and γ-secretase, the catalytic unit of which is presenilin. Findings reported during the late 1980s and early 1990s led to the proposal of the "Aβ cascade hypothesis" of AD onset, which states that Aβ generation is a primary cause of AD [3]. Several lines of evidence indicate that the amyloidogenic processing of APP, including Aβ generation, occurs in membrane microdomains termed lipid rafts [4]. However, the molecular mechanisms underlying APP translocation to lipid rafts remain unclear. In this chapter, regulatory mechanisms for lipid raft translocation of APP and APP C-terminal fragments (APP CTFs) generated primarily by the cleavage of APP are described.

Membrane lipid rafts are known as sites of amyloidogenic processing of APP and enriched with active β-secretase, while non-amyloidogenic cleavage of APP by α-secretase is performed outside lipid rafts. Neural adaptor protein X11-like (X11L) regulates the translocation of mature APP (mAPP), which is the *N*- and *O*-glycosylated form and real substrate of

secretases in the late protein secretory pathway, to lipid rafts. APP bound to X11L preferentially localizes to sites outside of lipid rafts and escapes from active β-secretase [5]. Dissociation of the APP-X11L complex leads to APP entry into lipid rafts, suggesting that dysfunction of X11L in its interaction with APP may recruit more APP to lipid rafts and increase the generation of Aβ [5].

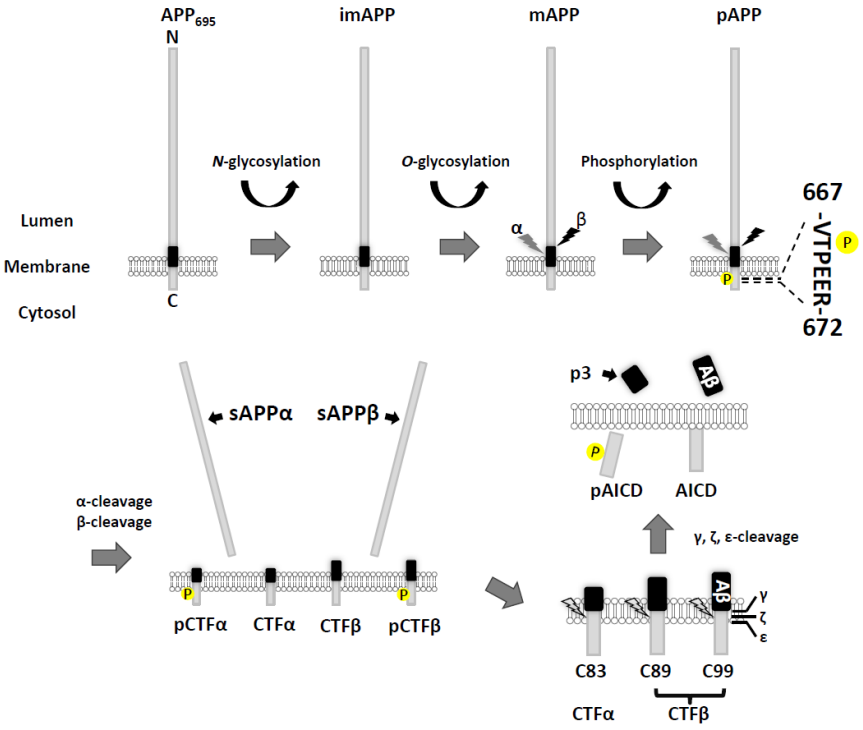

Figure 1. The schema of APP metabolism and post-translational modification of APP. APP is subjected to *N*-glycosylation at ER to form imAPP followed by *O*-glycosylation at the *medial-/trans*-Golgi apparatus to form mAPP. Residue Thr668 of mAPP is specifically phosphorylated in brain. mAPP is cleaved in sequential proteolytic events mediated by β-secretase or α-site APP cleaving enzyme, and the γ-secretase complex. β-secretase primarily cleaves APP in the luminal domain to generate sAPPβ and CTFβ (C99 and C89). C99 contains an intact Aβ sequence. γ-secretase complex mediates the cleavage of CTFβ at ε, ζ, and γ-sites to generate Aβ and AICD peptides. α-site APP cleaving enzyme generates sAPPα and CTFα (C83). CTFα cleavage by γ-secretase complex then generates p3 peptide and AICD.

In contrast to APP, APP CTF translocation to lipid rafts seems to involve another regulatory system that also includes active γ-secretase to cleave APP CTFs. The translocation of CTFs to lipid rafts is regulated by APP phosphorylation. The cytoplasmic region of APP is well known to demonstrate neuron-specific phosphorylation at Thr668 (numbering for the APP695 isoform). However, the maximum phosphorylation level of APP is 10–20% in the brain, and its physiological function is not clear [6].

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A recent study found that the phosphorylation level of APP CTFs was much higher than that of full-length APP, and phosphorylated CTFs (pCTFs), but not nonphosphorylated CTFs (nCTFs), were preferentially located outside of detergent-resistant, lipid raft-like membrane microdomains, indicating that Thr668 phosphorylation appears to function on the APP CTF rather than full-length APP [7]. Recent analysis revealed that pCTFs are relatively movable within the membrane as integral membrane proteins, while nCTFs are susceptible to being anchored to a lipid raft by direct binding of the C-terminal tail to membrane lipids. Once in lipid rafts, nCTFs can be preferentially captured and cleaved by γ-secretase. Interestingly, phosphorylation levels of amyloidogenic CTFβ were significantly decreased in aged brain [7]. Two molecular mechanisms of APP and APP CTF translocation to ripid rafts are described in the following section.

2. Metabolism and post-translational modification of APP

APP, which is a type I membrane protein, is subjected to *N*-glycosylation at the endoplasmic reticulum (ER) to form immature APP (imAPP) followed by *O*-glycosylation at the *medial-/trans*-Golgi apparatus to form mature APP (mAPP) (Figure 1). mAPP is then transported through the *trans*-Golgi network to the plasma membrane, where it enters the late secretory pathway and is metabolized by either amyloidogenic or amyloidolytic (non-amyloiodgenic) processing [6, 8]. In the amyloidogenic pathway, APP is cleaved in sequential proteolytic events mediated by β-secretase (β-site APP cleaving enzyme 1 or BACE1) and the γ-secretase complex comprised of four core subunits, presenilins (PS1 or PS2), anterior pharynx defective 1 (APH-1), presenilin enhancer 2 (PEN2), and nicastrin. β-secretase primarily cleaves APP in the luminal domain to generate soluble APPβ (sAPPβ) and membrane-associated APP carboxyl terminal fragments (CTFβ/C99 and CTFβ′/C89). C99 contains an intact Aβ sequence (Figure 1). γ-secretase complex mediates the cleavage of CTFβ at ε, ζ, and γ-sites to generate Aβ and APP intracellular domain (AICD) peptides. Non-amyloidogenic cleavage of APP is mediated by α-site APP cleaving enzyme (α-secretase, including ADAM9, ADAM10, and ADAM17) to generate sAPPα and CTFα (C83), which contains only the carboxyl half of Aβ peptide. CTFα cleavage by γ-secretase complex then generates p3 peptide and AICD.

Residue Thr668 of the APP cytoplasmic region is located within the 667-VTPEER-672 motif and is phosphorylated (number corresponding to the APP695 isoform) in the late secretory pathway in neurons. Protein kinases such as GSK3β (glycogen synthase kinase-3β), CDK5 (cyclin-dependent kinase-5), CDK1/CDC2, and JNK (c-Jun N-terminal kinase) are thought to mediate this phosphorylation of APP [6]. APP CTFs are also phosphorylated at Thr668 and detected as phosphopeptide pC99, pC89, and pC83 by western blot analysis using a phosphorylation-state-specific anti-APP Thr668 antibody or pAPP antibody (Figure 2A). Typical APP CTF species in the brain appear as five bands: pC99, nC99, pC89, a mixture of nC89 plus pC83, and nC83. Treatment of CTFs with phosphatase is effective for the identification of the respective species. Levels of the phosphorylated CTFβ species pC99 and pC89 were significantly higher than those of their nonphosphorylated forms, nC99 and nC89, while

phosphorylated CTFα, pC83, demonstrated a trend toward decreased levels in comparison to nonphosphorylated CTFα, nC83 (Figure 2B). The relative ratio of total phosphorylated CTFs was equivalent to that of nonphosphorylated CTFs (Figure 2C), although phosphorylated CTFβ and CTFβ' were predominant compared to their nonphosphorylated forms. These observations indicate that pCTFs and nCTFs are present at equal levels in the brain as potential substrates for γ-secretase.

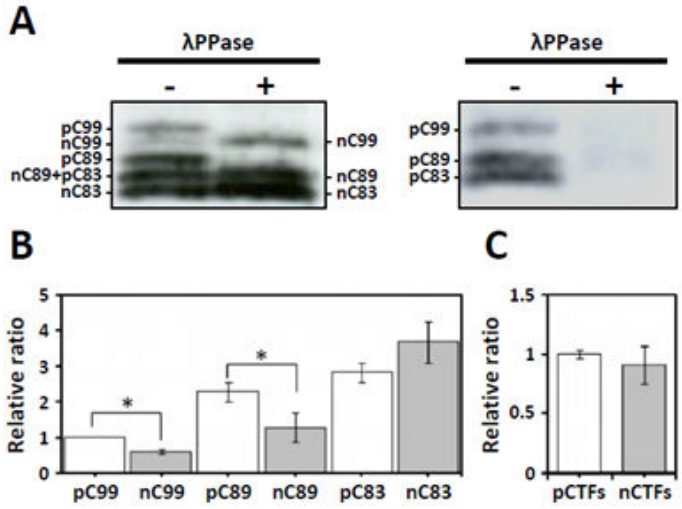

Figure 2. Level of CTF species in brain membrane fractions. (A) CTF species in brain membrane preparations. (B) and (C) Levels of CTF species in brain membrane preparations. Levels of the phosphorylated CTFβ species (pC99 and pC89) were significantly higher than those of their nonphosphorylated forms, nC99 and nC89.

The 667-VTPEER-672 motif, including the phosphorable amino acid Thr668, forms a type I β-turn and N-terminal helix-capping box structure to stabilize its C-terminal helix structure [9]. Therefore, phosphorylation of Thr668 induces significant conformational changes in the cytoplasmic region of APP (Figure 3) that affect its interaction with FE65, a neuronal adaptor protein [10]. The usual procedure to explore the function of a protein phosphorylation site is to mimic the phosphorylation state by amino acid substitutions of Asp or Glu for the appropriate Thr and Ser residues. However, this strategy may not be suitable in the case of APP phosphorylation, as the substitution of Asp for Thr668 did not alter the carboxyl terminal helix state as remarkably as phosphorylation of Thr668 (Figure 3A). By contrast, substitution of Thr668 with Ala668 in APP has been found to mimic effectively the nonphosphorylated state in the helix structure of the APP cytoplasmic domain. Figure 3B presents a schematic illustration of the Thr668-dependent conformational changes. Thr668Ala mutation mimics the nonphosphorylated state of APP, but Thr668Asp mutation did not completely mimic the phosphorylation structure of APP. Therefore, to reveal the role of APP phosphorylation at Thr668, careful analysis for the phosphorylation state of both APP and the APP metabolic fragments in the brain are described here.

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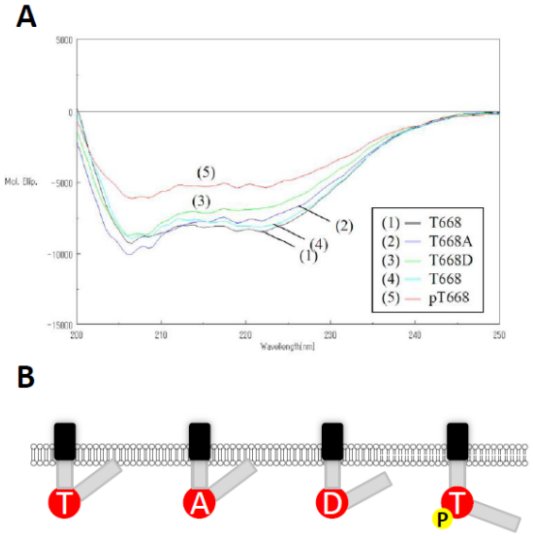

Figure 3. Circular dichroism (CD) spectra of APP cytoplasmic peptides (A) and schematic of changes to the APP cytoplasmic domain dependent on Thr668 residue modification (B). The substitution of Asp for Thr668 did not alter the carboxyl terminal helix state as remarkably as phosphorylation of Thr668. By contrast, substitution of Thr668 with Ala668 in APP has been found to mimic effectively the nonphosphorylated state in the helix structure of the APP cytoplasmic domain.

3. Lipid rafts and Alzheimer's disease

Dynamic and highly ordered membrane microdomains, termed lipid rafts, are rich in cholesterol and sphingolipids such as seramide, gangliosides, glycerophospholipids, and sterols. The average diameter of lipid rafts has been estimated at 50 nm. However, several classes of lipid rafts that vary in size and duration can exist in a cell [11]. Lipid rafts are formed in the Golgi and transported to the plasma membrane [12], where they serve as platforms for cell signaling, pathogen entry, cell adhesion, and protein sorting. Lipid rafts are biochemically defined as the detergent-resistant membrane (DRM) fraction [12]. Aβ generation and aggregation occur in lipid rafts, suggesting that lipid rafts play an important role in APP processing and subsequent AD pathogenesis. A growing body of evidence indicates that active β- and γ-secretases are located in membrane microdomains [13-15]. *S*-Palmitoylation of BACE1 at residues Cys474/478/482/485 is essential for the localization of BACE1 to lipid rafts [13,14]. *S*-Palmitoylation of nicastrin at Cys689 and of APH1 at Cys182 and Cys245 contributed to their stability and the lipid raft association of these nascent subunits, but did not affect the lipid raft localization of PS1 and PEN2 or the assembly of γ-secretase complex [15]. Taken together, lipid raft localization of secretases involved in amyloidogenic APP cleavage is regulated by their post-translational modification. However, the factors that determine lipid raft localization of APP remain unclear.

4. X11 protein regulation of APP localization to lipid rafts

X11 family proteins (X11s), consisting of X11/X11α/Mint1, X11L/X11β/Mint2, and X11L2/X11γ/Mint3, are encoded by separate genes on human chromosomes 9, 15, and 19 and mouse chromosomes 19, 7, and 10, respectively. X11s contain an evolutionarily conserved central phosphotyrosine binding/interaction (PTB/PI) domain and two C-terminal PDZ domains [16]. The PTB/PI and PDZ domains are well-characterized protein-protein interaction domains, and X11 proteins interact with various types of proteins, including APP, alcadein, apoER2, munc18, KIF17, kalirin, hyperpolarization-activated cyclic nucleotide-gated (HCN) channel, and Arfs, through their PTB/PI and PDZ domains. Interaction of X11L with APP can stabilize APP metabolism and intracellular trafficking, which induce the suppression of Aβ generation [16-18]. Metabolic analysis of APP in X11 and/or X11L knockout mice confirmed that X11s modulated APP metabolism and suppressed Aβ generation as an endophenotype *in vivo* [5, 19, 20]. X11 or X11L transgenic mice crossed to commonly used AD model mice (APPswe transgenic mice) demonstrated reduced amyloid deposition along with decreased levels of Aβ40 and Aβ42 in the brain compared to APPswe transgenic mice [21, 22].

The molecular mechanisms underlying the suppression of APP amyloidogenic metabolism by X11 and X11L have been addressed in a recent analysis. In the brains of mice lacking X11 and/or X11L, levels of CTFβ and Aβ were increased relative to wild-type animals (Figure 4) [5].

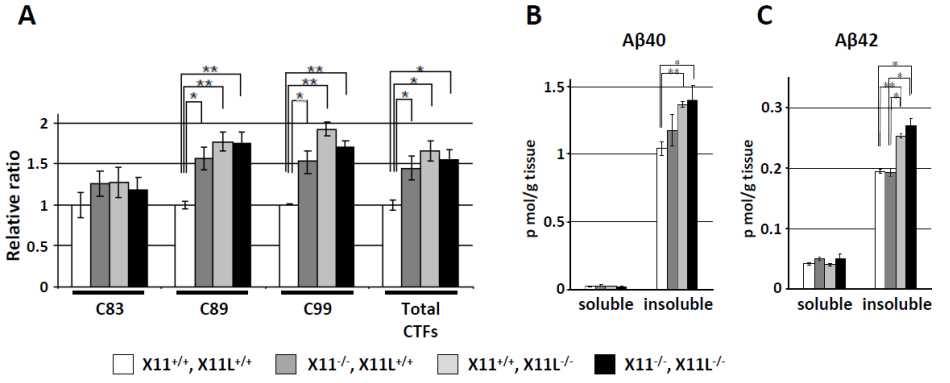

Figure 4. Quantification of APP CTFs in the hippocampus of wild-type, X11-deficient, X11L-deficient, and X11/X11L doubly deficient mice. Levels of CTFβ and Aβ were increased in X11s deficient mice, indicating that amyloidogenic metabolism of APP was enhanced in X11s deficient mice.

The absence of X11s resulted in more APP and APP CTF translocation to DRMs and enhanced colocalization of APP or APP CTFs with BACE1 in DRMs but not in non-DRMs (Figure 5A and B) [5]. Interestingly, X11s were recovered in membrane fractions, and they largely localized to non-DRMs but not DRMs (Figure 5C), indicating that APP can associate exclusively with X11s outside of DRMs to prevent APP translocation to lipid rafts, where amyloidogenic metabolism of APP occurs (Figure 6).

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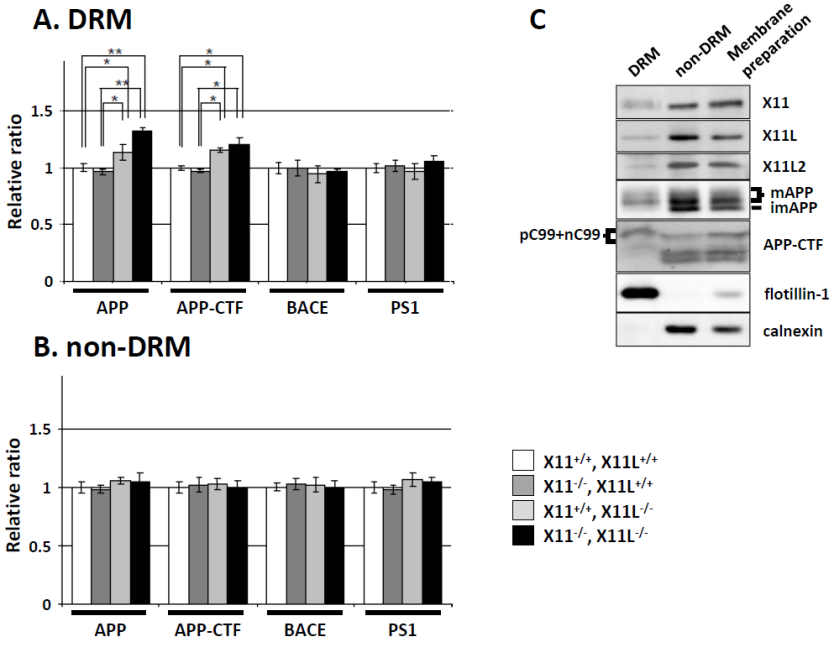

Figure 5. Quantification of APP, APP CTFs, BACE, and PS1 in (A) DRM and (B) non-DRM fractions from wild-type, X11-deficient, X11L-deficient, and X11/X11L doubly deficient mouse cortex. Higher levels of mAPP and CTFβ were recovered in DRM of the X11L-deficient and the X11/X11L doubly deficient mouse brain. (C) Localization of membrane-attached X11 proteins to DRM and non-DRM fractions. X11s were recovered in membrane fractions, and they largely localized to non-DRMs but not DRMs.

The Dysfunction of X11s in aged neurons may thus contribute to sporadic AD etiology. The dysfunction of X11s could lead to a weakening of the association between X11s and APP, resulting in greater translocation of APP to DRMs. Alteration in the lipid composition of membranes may enlarge lipid raft areas or increase the number of lipid rafts, which could also enhance APP translocation to DRMs. These qualitative alterations in X11s and/or lipid metabolism could result in increased β-cleavage of APP even if β-secretase itself is not enzymopathic.

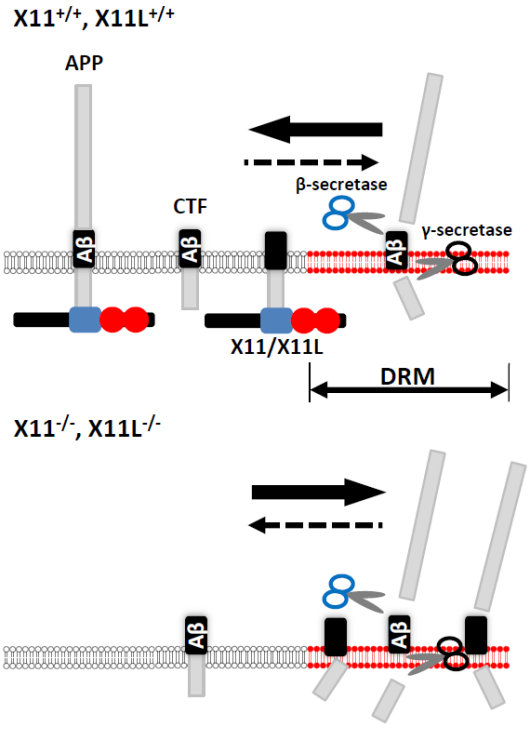

Figure 6. Possible role of X11 proteins in regulating the DRM association and β-site cleavage of APP. X11s associate with APP outside of DRMs and prevent translocation of APP into DRM. When X11L dissociates from APP, the APP translocates into DRMs, and that fraction of APP molecules is cleaved by BACE which is active in DRM (upper panel). In the absence of X11s, APP molecules are not anchored outside of DRMs, and more APP translocates into DRMs, resulting in increased β-site cleavage of APP (lower panel). The arrows indicate translocation direction of APP.

5. Regulation of APP CTF translocation to lipid rafts by Thr668 phosphorylation

Because similar amounts of nCTFs and pCTFs were found in mouse brain (Figure 2C), generation of similar levels of the APP intracellular cytoplasmic domain fragments, nonphosphorylated AICD (nAICD) and phosphorylated AICD (pAICD), is expected if γ-secretase cleaves nCTFs and pCTFs equivalently. However, membrane prepared from mouse brain generated higher levels of nAICD than pAICD in an *in vitro* γ-secretase assay (Figure 7A). Incubation of membrane preparations demonstrated a time-dependent, nearly linear increase in the generation of nAICD and pAICD during the 0–2 h time period, and the reaction essentially reached a plateau in the 2–4 h period (Figure 7B and C). Dephosphorylation and degradation of pAICD did not occur in this assay. Importantly, the ratio of pAICD to AICD generation was constant throughout the incubation time (1–4 h) with the relative ratio

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(amount of pAICD/amount of nAICD) measuring 0.35 ± 0.10 at the 2 h point (Figure 7D). Taken together, these *in vitro* analyses indicate that both phosphorylated and nonphosphorylated CTFs are kinetically equivalent as a substrate for γ-secretase, but the results also show that the generation of pAICD was significantly lower when compared to that of nAICD. These observations suggest that pCTFs are located at a distance from active γ-secretase in the membrane, while nCTFs are positioned nearer to the active enzyme.

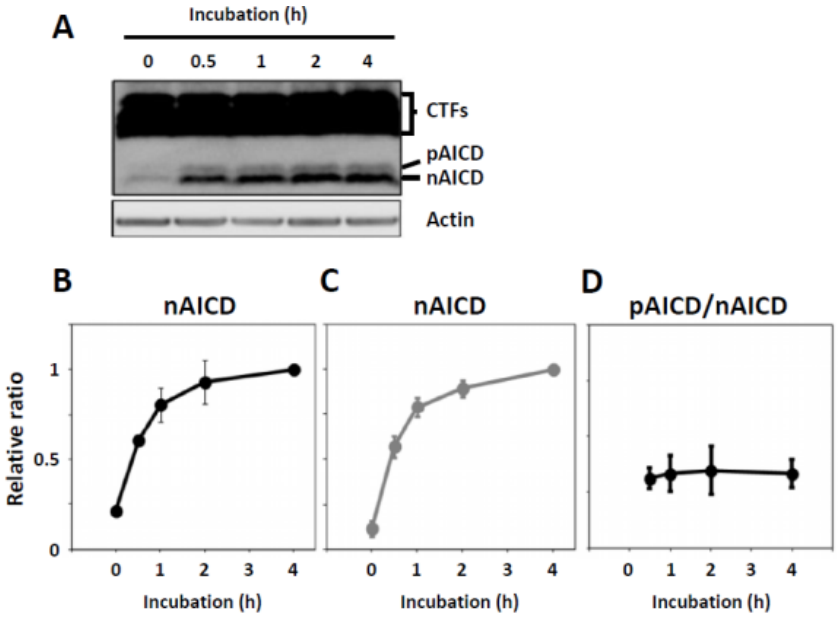

Figure 7. *In vitro* kinetic analysis of phosphorylated and nonphosphorylated CTF cleavage by γ-secretase. (A) *in vitro* γ-secretase assay with membrane preparations from wild-type mouse brain. (B) and (C) kinetic analysis of AICD generated by incubation of membrane preparations. (D) the production ratio of pAICD to nAICD (pAICD/nAICD) at the indicated times are shown. Both phosphorylated and nonphosphorylated CTFs are kinetically equivalent as a substrate for γ-secretase, but the results also show that the generation of pAICD was significantly lower when compared to that of nAICD.

Thr668 phosphorylation could regulate APP CTF translocation to the lipid raft microdomain. To examine this hypothesis, γ-secretase-enriched lipid raft-like membrane microdomains were prepared as DRMs using CHAPSO. Application of CHAPSO is preferable for the isolation of DRMs, including active γ-secretase complexes, compared to procedures using other detergents such as Triton X-100 [23, 24]. Components of the active γ-secretase complex, both PS1 N- and C-terminal fragments and PEN2, were predominantly recovered in the DRM fraction along with a small amount of APP CTFs (~20% measured) [7]. Phosphorylation levels of APP CTFs in the DRM and non-DRM fractions were examined, and the respective nCTFs and pCTFs were compared as a relative ratio in which pC99 in the DRM was set to 1.0 (Figure 8).

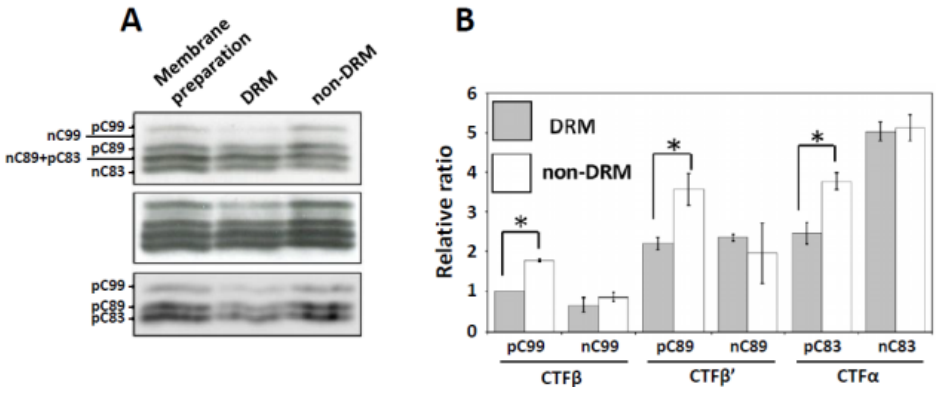

Figure 8. Quantification of pCTFs and nCTFs in DRM and non-DRM fractions. (A) Identification of APP CTFs in DRM and non-DRM fractions. (B) CTFs levels in DRM and non-DRM fractions. Significantly higher levels of the phosphorylated species pC99, pC89, and pC83 were found in the non-DRM fractions.

Significantly higher levels of the phosphorylated species pC99, pC89, and pC83 were found in the non-DRM fractions compared to the DRM fractions. Additionally, the phosphorylation level of total APP CTFs in DRM was significantly lower than that in non-DRM. These results indicate that phosphorylated CTFs are preferentially localized outside of the DRM/lipid raft-like membrane microdomain and thus prevented from cleavage by γ-secretase.

How does phosphorylation of Thr668 regulate the localization of APP CTFs between DRM and non-DRM? A recent structural analysis revealed that the cytoplasmic domain tail of APP can interact with membrane lipids [25]. Since phosphorylation of APP at Thr668 induces a significant change in its cytoplasmic domain conformation (Figure 2) [9, 10, 26], phosphorylation of the APP cytoplasmic domain at Thr668 can influence the association of the APP cytoplasmic tail with membrane lipids.

Liposomes prepared with endogenous lipids from the membrane fractions of mouse brain have been used as a model for neural membranes [27]. Synthetic cytoplasmic APP 648–695 peptide with (pC47) or without (nC47) a phosphate group at residue Thr668 was incubated with the liposomes, and the liposome-bound peptides were recovered and analyzed by immunoblotting. Notably, nonphosphorylated APP cytoplasmic peptide (nC47) bound strongly to the liposomes, while phosphorylated peptide (pC47) demonstrated no detectable association (Figure 9A) [7]. This trend was also confirmed by examining the AICD, which lacks the transmembrane domain due to ε-cleavage by γ-secretase [28, 29]. Most nAICD was recovered in the brain membrane fraction (~75%) rather than in the soluble cytoplasmic fraction (~25%), while comparatively more pAICD was found in the cytoplasmic fraction (~45%) (Figure 9B).

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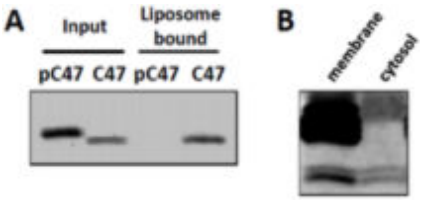

Figure 9. Liposome-binding ability of APP cytoplasmic domain and ist localization in mouse brain. (A) The binding ability of the phosphorylated APP cytoplasmic domain peptide with liposomes composed of lipids from mouse brain membranes. (B) distribution of AICD endogenously generated in mouse brain. Nonphosphorylated nC47 and AICD bound strongly to the liposome and membrane fraction.

Therefore, the nonphosphorylated forms of APP CTFs and AICD tend to bind membrane lipids, mediated by their C-termini, and phosphorylation of APP CTFs and AICD at Thr668 functions to prevent direct membrane association, apparently by changing the conformation of their cytoplasmic regions. In addition to these observations, pCTFβ levels were significantly decreased with age in cynomolgus monkey brains [7], indicating that the preservation of APP CTF phosphorylation levels correlates with the suppression of γ-cleavage.

To conclude this section, first, almost equal amounts of pCTFs and nCTFs are present in mouse brain, while lower amounts of pAICD are generated compared to nAICD. Second, both pAICD and nAICD are kinetically equivalent substrates for γ-secretase. These observations suggest that pCTFs are sequestered away from the membrane region where γ-secretase is active (DRM/lipid raft-like membrane microdomain) [15], and that pCTFs are located outside of the DRM/lipid raft-like membrane microdomain due to a change in the conformation of their cytoplasmic tail, to which the membrane lipids bind. Thus, the pCTFs can quickly disperse from the DRM/lipid raft-like membrane microdomain with their increased mobility in the membrane (Figure 10).

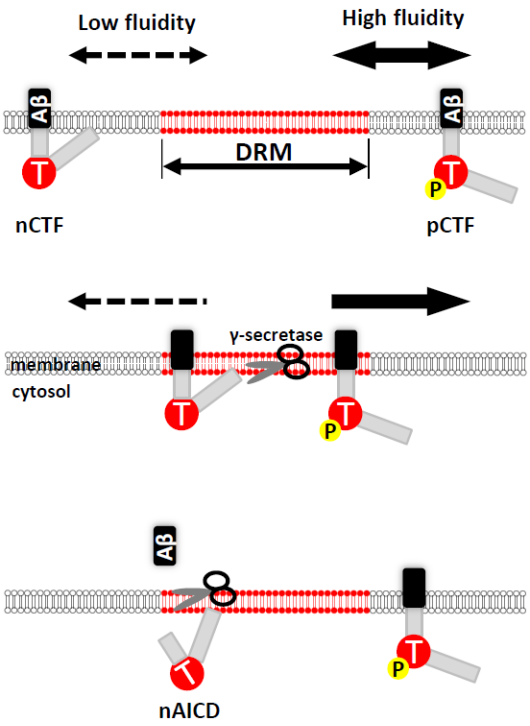

Figure 10. Possible role of APP CTF phosphorylation at Thr668 in regulating its fluidity within the membrane and its cleavage by γ-secretase.

6. Conclusions

X11L abundantly present in non-DRM traps APP outside of the DRM and prevents contact between APP and the β-secretases located within the DRM. Phosphorylation of APP at Thr668 induces conformational changes to the APP cytoplasmic domain and reduces the affinity of the APP C terminal to lipids. This change alters APP CTF fluidity and decreases the probability of APP CTF presence in lipid rafts, in which contact between APP CTFs and γ-secretase occurs. In conclusion, translocation of APP and APP CTFs to lipid rafts is regulated by neuronal adaptor protein X11L and Thr668 phosphorylation of APP CTFs.

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Abbrevations

ADAM: a disintegrin and metalloprotease domain, APH-1: anterior pharynx defective 1, AICD: APP intracellular domain, APP: amyloid precursor protein, APP CTFs: APP C-terminal fragments, BACE1: β-site APP cleaving enzyme 1/β-secretase, CDK5: cyclin-dependent kinase-5, CD spectra: Circular dichroism spectra, DRM: detergent-resistant membrane, GSK3:βglycogen synthase kinase-3β, JNK: c-Jun N-terminal kinase, imAPP: immature APP, mAPP: mature APP, pAICD; nAICD; nonphosphorylated AICD, nCTFs; nonphosphorylated CTFs, phosphorylated AICD, pCTFs; phosphorylated CTFs, PS: presenilins, PEN2; presenilin enhancer 2, PTB/PI domain; phosphotyrosine binding/interaction domain; sAPP; soluble APP, X11L; X11-like.

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