JB Minireview—Membrane Traffic in Physiology and Pathology

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Trafficking of Alzheimer's Disease–Related Membrane Proteins and Its Participation in Disease Pathogenesis

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Alzheimer's disease (AD) is a common neurodegenerative disorder that causes senile dementia. The pathological characteristics are the appearance of neurofibrillary tangles comprising abnormally phosphorylated tau and senile plaques composed of amyloid β-protein depositions. Amyloid β-protein precursor (APP) and presenilin (PS) are known to be causative genes of familial AD. Recent analyses have documented that APP functions in the axonal transport of vesicles and PS regulates intracellular protein trafficking. Dystrophic neurites, in which APP and Alcadein accumulate in swollen axons, are also observed in AD brain. These pathological characteristics and the features of AD-related proteins suggest that AD is a disease of the vesicular transport system. Here we review recent progress of research on AD pathogenesis from the viewpoint of membrane trafficking.

Key words: Alzheimer's disease, amyloid β-protein precursor, cargo-receptor, kinesin, membrane trafficking, neurodegeneration.

Terminally differentiated neurons have specialized shapes, usually including a long axon and a highly branched, complex dendritic tree. A large nucleus and a substantial amount of protein synthetic machinery are needed to maintain the structures and functions of neurons with such large cellular volumes. To maintain this functional architecture, which arises from a well-organized collaboration of membrane and cytoskeletal components, neurons are one of typical cells that have developed a system of intracellular movement of materials, the "membrane trafficking" system. Disorders of this intracellular material transport system cause degeneration of neural function and lead to neuronal death, resulting in "neurodegenerative disease."

Alzheimer's disease as a disease of neurodegeneration and membrane trafficking

Aberrant or deficient axonal transport in neurons is observed in a variety of neurodegenerative diseases. Pathologically, these disorders can appear as axonal swellings, which are formed as a result of the unusual accumulation of cargo-related proteins (*1*, *2*). AD, the most common neurodegenerative form of senile dementia, causes progressive defects of cognition and loss of neurons. The main pathological characteristics of AD are the appearance of neurofibrillary tangles and senile plaques (*3*). Neurofibrillary tangles are observed within cells; paired helical filaments, which are composed of abnormally phosphorylated tau protein, are their major component. The neurofibrillary tangles in neurons arise from a disorder of the cytoskeleton system that generates a defect in the microtubule-dependent vesicular transport system. Senile plaques are extracellular depositions of amyloid β-protein (Aβ), which is derived from proteolytic cleavage of the amyloid β-protein precursor (APP), a causative gene product of familial Alzheimer's disease (FAD). Dystrophic neurites and axonal swelling are also observed around the senile plaques, and immunostaining for APP in dystrophic neurites is a sensitive method for detecting disturbances in axonal transport in the AD brain (*4*) (Fig. 1). The disruption of the vesicular transport system observed in AD brains suggests that defects in the membrane trafficking system, including axonal vesicular transport, may be a primitive cause of AD pathogenesis. Therefore, elucidating the molecular mechanism of trafficking of membrane-associated proteins is important for understanding AD pathogenesis.

Transport, metabolism, and function of membrane components associated with AD

Several membrane and transmembrane proteins have been associated with AD, including APP, Alcadein, the ADAM family of proteins, BACE, and the proteins of the γ-secretase complex.

APP. APP is a type I transmembrane protein. Three major isoforms of 695, 751, and 770 amino acids are encoded by single a gene. The major isoform of APP in neurons is APP695. Newly synthesized APP is transported through the protein secretory pathway (Fig. 2). APP is subject to *N*-glycosylation in the ER and *O*-glycosylation in the Golgi apparatus; then the membrane vesicles containing APP are transported along the axon and are subsequently transcytosed to localize in dendrites (*5*). APP is exposed on the plasma membrane and some is internalized into endosomes for recycle. APP is thought to be subjected to proteolytic processing in the secretory pathway, on the plasma membrane, and/or in the endocytotic cycle. APP is

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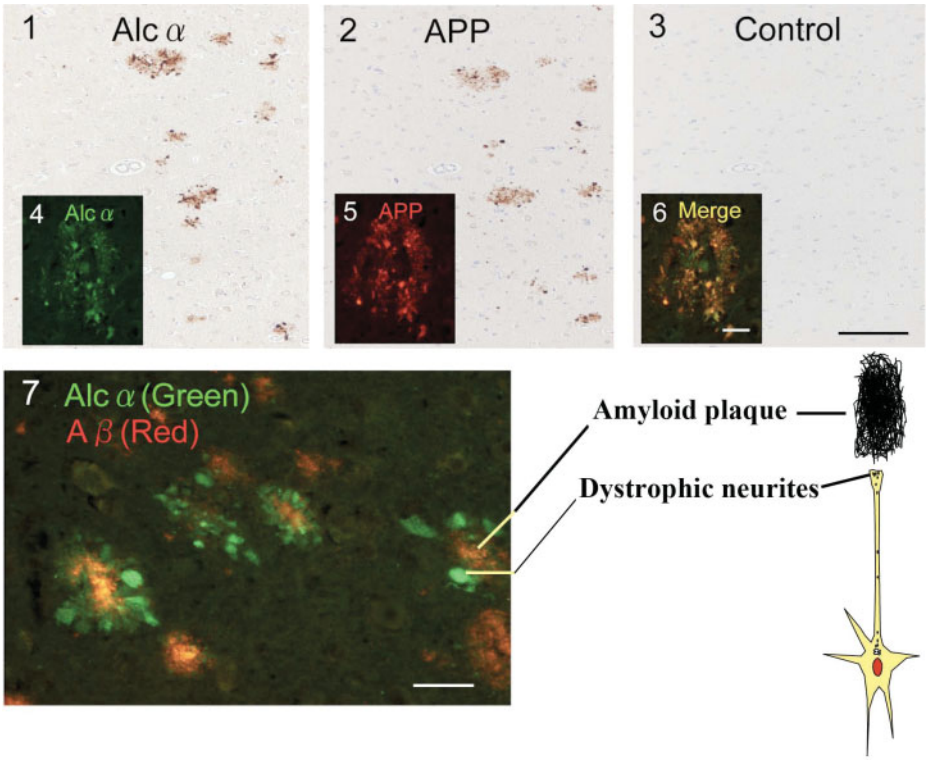

Fig. 1. **Localization of Alcadeinα (Alcα), APP, and β-amyloid (Aβ) in the brain of an Alzheimer's disease patient.** Panels 1–3, immunostaining with anti-Alcα (1), anti-APP (2), and non-immune (3) antibodies. Panels 4–6, double immunofluorescence staining for Alcα (4) and APP (5), and their merged image (6). Panel 7, double immunofluorescence staining for Alcα and Aβ. Scale bar, 20 μm (white) and 100 μm (black). Alcα and APP are localized in the dystrophic neurites of disseminated senile (amyloid) plaques, which are indicated by the staining of Aβ. [Modified with permission from The American Society for Biochemistry & Molecular Biology (*24*)]

cleaved consecutively by α-, β-, and γ-secretases. Although the exact place in the cell where this cleavage occurs is still unclear, it is believed that the metabolism of APP is affected by state of the protein secretory pathway (Figs. 2 and 3A). Qualitative and quantitative alterations of APP metabolism, such as increased generation of Aβ42, a longer, pathogenic form of Aβ, are believed to be facilitated by defects in the protein secretory pathway. Moreover, a report that axonal defects are present in patients in the early stages of AD supports the idea that axonopathy and transport deficits of neurons, as well as Aβ generation, are related to pathogenesis of AD (*6*).

The APP cleaving enzymes ADAM10 and ADAM17 (α-secretase) (*7*, *8*); BACE (β-secretase) (*9*); and the γ-secretase complex composed of presenilin (PS), nicastrin (NCT), APH-1, and PEN-2 (*10*), are all membrane-associated proteins and are also distributed through vesicular trafficking. Because Aβ is generated when APP is cleaved by the combination of β- and γ-secretases (Fig. 3A), a current focus of research is whether APP is co-transported with BACE and the γ-secretase complex in the same vesicle (*11*, *12*).

The transport, metabolism, and function of APP are regulated by interactions with several cytoplasmic proteins. APP possesses a short cytoplasmic tail containing several functional motifs: 653-YTSI-656, 667-VTPEER-672, and 681-GYENPTY-687 (amino acid numbering for the APP695 isoform) (Fig. 3B). Various cytoplasmic adaptor proteins bind to the 681-GYENPTY-687 motifs. The 667-VTPEER-672 motif regulates the α-helical structure of the overall cytoplasmic region by forming an amino-terminal helix capping-box structure that helps to stabilize the neighboring carboxyl-terminal helix structure (*13*). Relatively well analyzed APP-binding partners include FE65, X11 and X11L, and JIP1b. FE65, X11, and X11L are neural-specific adaptor proteins (*14–16*) (Fig. 3B).

Recent analyses revealed that FE65 can function in gene transactivation mediated by the APP intracellular domain (AICD), the cytoplasmic fragment liberated by consecutive cleavages of APP (*17*) (Fig. 3). Targeted deletion of two members of the FE65 family, FE65 and FE65L1, produce phenotypes similar to those observed in triple knockout mice lacking the APP family members APP, APP-like protein 1 (APLP1), and APP-like protein 2 (APLP2) (*18*, *19*). This suggests that APP works as a component of signal transduction pathways. Phosphorylation of Thr668 in the 667-VTPEER-672 motif induces a conformational change in the APP cytoplasmic domain, including the FE65-binding motif, 681-GYENTPY-687, and suppresses the interaction of APP and AICD with FE65 (*20*). The phosphorylation may work as a molecular switch in APP-FE65 signal transduction (*21*).

X11 and X11L regulate APP metabolism (*15*, *22*, *23*). In neurons, APP interacts with X11 or X11L to form a tripartite complex with Alcadein, a type I membrane protein, in Golgi or on plasma membrane but not during vesicular transport (*24*). APP is metabolically stable when in the tripartite complex, but naked APP is quickly degraded along with Alcadein in cells (*25*). Transgenic overexpression of X11 or X11L can suppress cerebral Aβ levels in the brains of transgenic mice expressing human APP carrying a familial AD mutation (*26*, *27*).

JIP1b is a JNK-interacting protein that bridges the association of APP with the kinesin I light chain (KLC) (*16*). Therefore, JIP1b is thought to be the most important adaptor protein in APP vesicular transport. FE65, X11 and X11L, and JIP1b each contain a phosphotyrosine interaction domain, to which the 681-GYENPTY-687

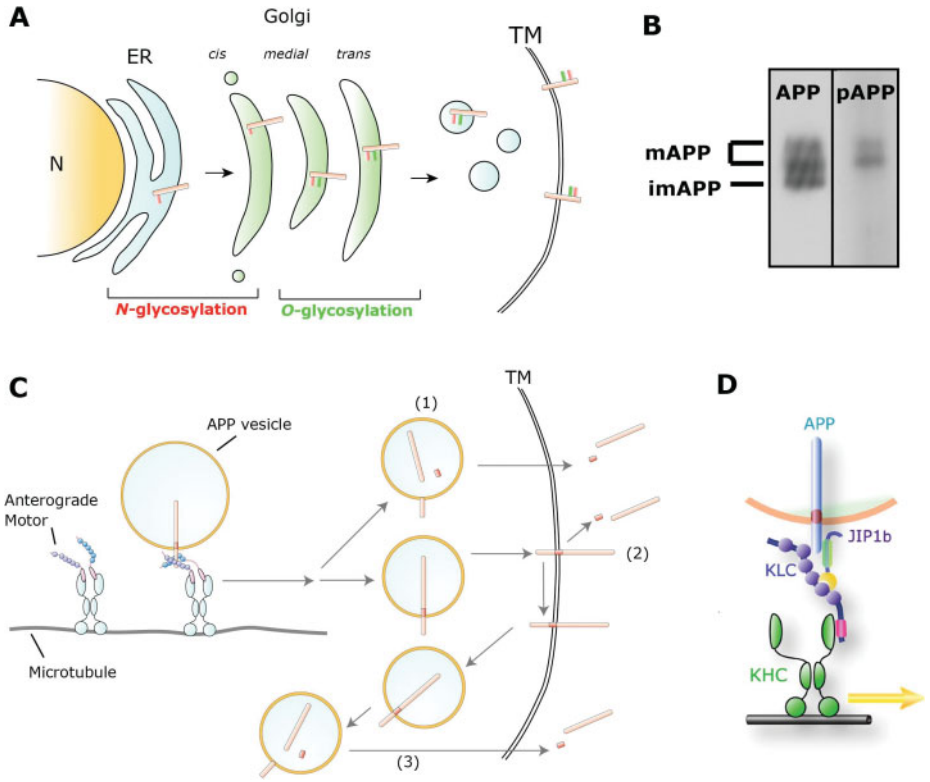

Fig. 2. **Membrane trafficking of APP-cargo.** A: APP in the classical secretory pathway. APP is subjected to *N*-glycosylation in the ER, *O*-glycosylation in the Golgi apparatus, and then reaches the plasma membrane. B: APP695 in mouse cortical neurons. The APP695 isoform is the predominant form in neurons. The *N*-glycosylated form is designated immature APP (imAPP) and the *N*- and *O*-glycosylated form is designated mature APP (mAPP). Different *O*-glycosylation results in two mAPP proteins that are subjected to phosphorylation at threonine 668 in the cytoplasmic domain (pAPP). C: Anterograde transport of APP vesicles and metabolism of APP. APP is subjected to cleavage during axonal transport (1), on the plasma membrane (2), or in the endocytotic cycle (3), generating neurotoxic Aβ. D: Association of APP with the kinesin-I motor. APP binds to KLC through an association with JIP1b, but does not bind to KLC directly. Thus, APP serves as a cargo-receptor *via* JIP1b.

motif of APP associates in a tyrosine phosphorylation-independent manner (*28*) (Fig. 3B).

Recent data suggest that APP is not only transported as cargo but also functions as a cargo receptor for kinesin-I to mediate the targeting of proteins to the nerve terminal. However, whether APP does in fact serve as a kinesin-I cargo receptor and what the cargo proteins are is still under consideration, as described below. APP can associate with KLC only when the JIP1b protein binds to both proteins (Fig. 2D), and APPL, a *Drosophila* homologue of APP (*29*), also associates with KLC through APLIP1, a *Drosophila* homologue of JIP1b (*16*).

Alcadein. The Alcadein (Alc) family of type I transmembrane proteins is composed of Alcadein α (Alcα), β (Alcβ), and γ (Alcγ), which are independent gene products. The human Alcs contain 981 (Alcα1), 968 (Alcβ), and 955 (Alcγ) amino acids, and Alcα1 has a splice variant of 991 amino acids (Alcα2). Alc-like genes are conserved in *D. melanogaster* and *C. elegans*, as is APP. The overall structure of the Alc family members is well conserved, and the amino acid sequences of the single cytoplasmic domain containing the X11/X11L-binding site and the acidic region are highly conserved (*24*). Alc is not thought to be a causative gene of FAD, but the localization and metabolism of Alc is extremely similar to that of APP. Alc and APP form a complex through cytoplasmic interactions with the X11/X11L protein in neurons, as described in the "APP" section above. Alc is also subject to primary cleavage at the extracellular juxtamembrane region prior to the second intramembrane cleavage by γ-secretase, although Alc is very stable when in a complex, as is APP (*25*). The coordinated metabolism of Alc and APP suggests that Alc functions physiologically in conjunction with APP. In fact, the AICD of APP is known to work in gene transactivation and AlcαICD, a cytoplasmic domain fragment liberated by consecutive cleavages of Alcα, regulates the FE65-dependent gene transactivation mediated by AICD (*25*). In AD brains, Alcα, like APP, accumulates in dystrophic neurites with axonal swellings (Fig. 1) (*24*). This suggests that the vesicular transport of the Alc holoprotein is subject to regulation similar to that of APP.

ADAM family. ADAM (a disintegrin and metalloprotease) is a type I transmembrane protein that functions as a zinc-dependent protease and is related to cell adhesion. More than 30 ADAM orthologues have been found in many species (*7*). Biosynthesized ADAM is transported to the Golgi apparatus, where its pro-domain is cleaved by furin or a furin-like protease, which activates the protease activity. After this cleavage, ADAM localizes at the cell surface, where it has been shown to cleave many cell adhesion molecules, growth factors, and growth factor receptors (*8*). Two of the proteins of this family, ADAM10 and ADAM17, are candidates for the α-secretase that cleaves APP at the α-site. Cholesterol depletion experiments showed that α-secretases, especially ADAM10, are active at non-lipid raft sites (*30*). Knock-out of each of these ADAM genes in mice reduces but does not completely abolish α-site cleavage. These two ADAMs are expressed in brain, but ADAM10 is expressed in neurons while ADAM17 is mainly expressed in glial cells. ADAM10 is a homologue of *Drosophila* Kuzbanian, which is an important molecule for axonal guidance (*31*). ADAM10 is also involved in regulation of neurite outgrowth in mammalian cells (*32*), and ADAM10-deficient mice die with multiple dysfunctions including CNS deficits (*33*).

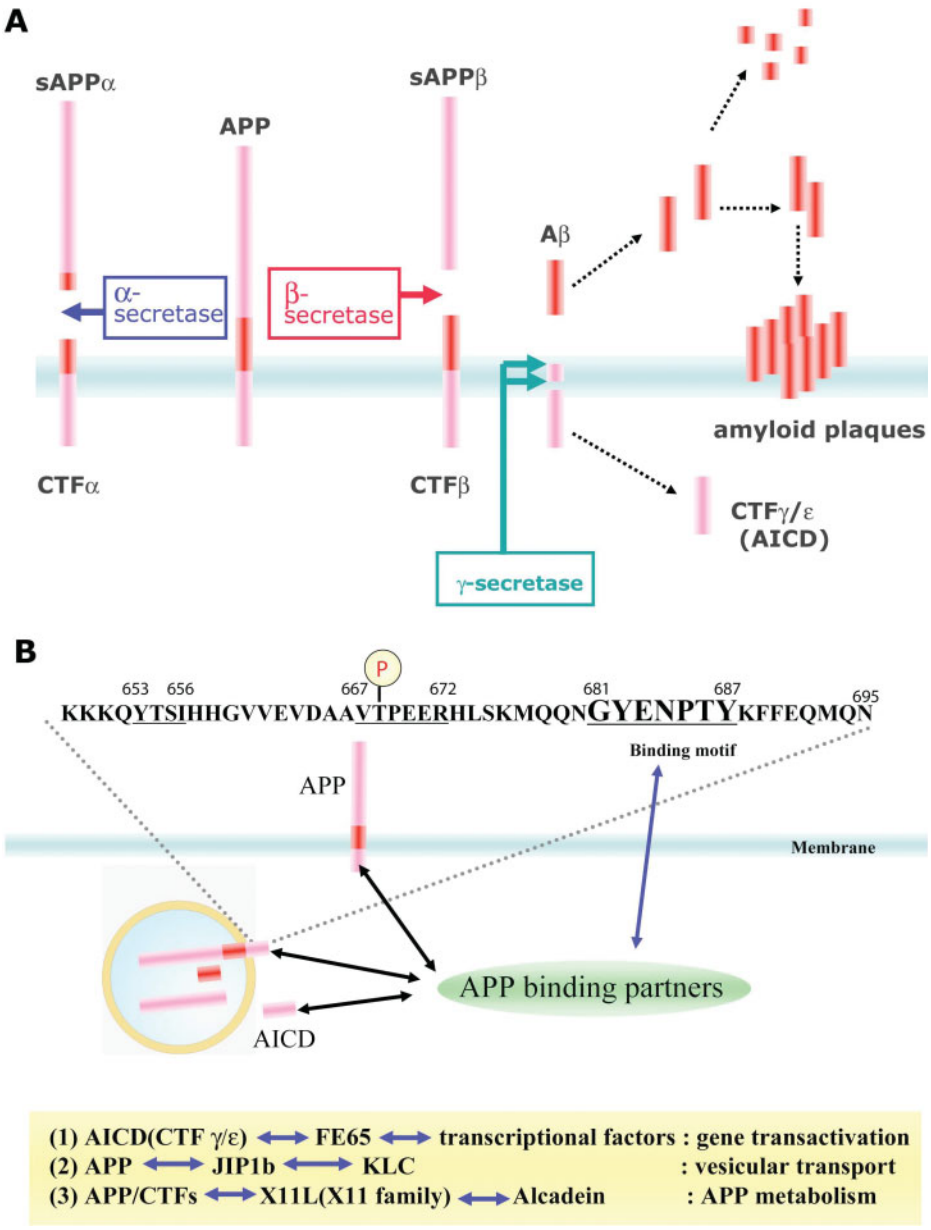

Fig. 3. **Schematic diagram of APP metabolism, amino acid sequence of the cytoplasmic domain of APP, and its binding partners.** A: APP metabolism. The majority of mAPP is cleaved at the α-site by ADAM10 and ADAM17 (α-secretase), leading to shedding of the APP ectodomain sAPPα. A minority of mAPP is cleaved at the β-site by BACE (β-secretase), leading to shedding of the APP ectodomain sAPPβ. The carboxyl-terminal fragments generated by these primary cleavages, CTFα and CTFβ, are subjected to further intramembrane cleavages by γ-secretase at the γ- or ε-site. This generates Aβ from CTFβ, and the cytoplasmic fragment, CTFγ/ε (AICD). B: Motifs and APP-binding partners. The cytoplasmic domain of APP contains several functional motifs (underlined). Several cytoplasmic proteins (APP binding partners), such as FE65, JIP1b, and X11/X11L, bind to the 681-GYENPTY-687 motif. FE65 associates with AICD and is thought to serve in gene transactivation. JIP1b associates with APP and serves as a bridge to the kinesin-I motor (see Fig. 2D). The X11 and X11L proteins associate with APP and regulates metabolism and localization of APP. APP associates with Alc in neurons *via* X11L, thus forming a tripartite complex with X11L bridging the cytoplasmic tails of APP and Alc.

BACE. BACE (beta-site APP-cleaving enzyme) is a type I transmembrane aspartic protease and has two homologues, BACE1 and BACE2. BACE1-deficient mice do not produce any Aβ in neurons, but small amounts of Aβ remain in glial cells. In the brains of BACE1/BACE2 double-deficient mice, Aβ production is completely abolished even in glial cells (34). Thus, BACE1 is the major β-secretase of APP in neurons (Fig. 3A). Biosynthesized BACE1 is transported to the Golgi apparatus and subjected to pro-domain cleavage by a calcium-dependent furin-like protease, *N*-glycosylation, phosphorylation at the cytoplasmic domain, and transport to the cell surface. This maturation step in the vesicular transport system requires the cytoplasmic domain (*35*). After BACE1 is localized at the cell surface, it is internalized into endosomes. The cytoplasmic phosphorylation regulates the distribution into early or later endosomes (*36*). The addition of cholesterol depletion reagents to the media of cell linesin culture reduces β-secretase activity, suggesting that BACE1 functions at lipid raft sites (*30*). β-secretase activity is also observed in the endosome (*37*), indicating that acidic pH is optimal for BACE1 activity (*9*). Thus, mature BACE1 is likely transported to endosomal rafts to cleave APP at the β-site. However, in neurons, it is unclear whether BACE1 is usually transported through axons to nerve termini or is mainly transported to the somatodendritic compartment (*11*, *12*). BACE1 activity has been reported to increase in aged brains and the brains of AD patients (*38*, *39*).

γ-secretase complex. Patients with early-onset FAD have mutations in PS genes as well as the APP gene. Pathogenic mutations of presenilin 1 (PS1) and presenilin 2 (PS2) increase the ratio of Aβ42, the more aggregative and pathogenic Aβ, to Aβ40 (3). PS, a unique membrane-bound aspartic protease, is a membrane

protein possessing eight transmembrane regions and the catalytic unit of γ-secretase. The γ-secretase complex requires at least three other components. Human NCT is a type I membrane protein comprising 709 amino acids, APH-1 is membrane protein with four transmembrane regions, and PEN-2 is membrane protein with two transmembrane regions. All components known at present are conserved in *D. melanogaster* and *C. elegans*. These membrane-associated components are assembled in a stepwise, well ordered fashion during membrane trafficking through the ER and Golgi apparatus to form the active γ-secretase complex (*10*). Naked PS is very metabolically unstable and is rapidly degraded during trafficking, but PS is stable in high-molecular-weight complexes containing NCT and APH-1, and the addition of PEN-2 is thought to activate the γ-secretase activity. Recent observations have indicated that NCT functions as a γ-secretase-substrate receptor (*40*). PS has been implicated in regulating intracellular protein trafficking and turnover (*41–43*). Thus, the regulation of membrane trafficking of vesicles containing γ-secretase components and the assembly of components in the membrane are very important for physiological events related to γ-cleavage of type I membrane proteins. At present, over 30 type I membrane proteins, including Notch, Delta, CD44, APP, Alc, and *N*-cadherin, have been identified as substrates of γ-secretase (*44*). Mice lacking PS genes die embryonically (*45*).

Membrane trafficking of APP-containing vesicles

The first indication that APP undergoes fast anterograde axonal transport in neurons was derived from a study using a ligated sciatic nerve of rat (*5*). Substantial amounts of APP are detected in DRG neurons and the proximal sciatic nerve. When the sciatic nerve was ligated at a more distal region, APP accumulated progressively in the nerve segment immediately proximal to the ligature. Overexpression of APPL in *Drosophila* causes a defect in eclosed adults, and this phenotype is enhanced when the dosage of the kinesin heavy chain is reduced (*46*); this finding prompted the hypothesis that APP functions as a vesicular receptor for the kinesin motor. This hypothesis gained support when human APP was expressed in fly (*47*), and direct interaction of APP with kinesin-I was reported (*48*). The same research group also showed that an axonal membrane compartment whose fast axonal transport is mediated by APP contains BACE and PS1, APP cleaving enzymes that generate Aβ (*11*).

However, different research groups question the hypotheses that APP serves as a kinesin-I receptor and that the proteolytic processing machinery responsible for generating Aβ is transported in the same vesicular component in axons as APP (*12*). What is true about APP function in membrane trafficking and what should be reconsidered?

First, further analyses have clearly indicated that APP does not bind directly to KLC. Thus, the previous conclusion that APP forms a complex with conventional kinesin (kinesin-I) by binding directly to the tetratricopeptide repeat (TPR) domain of KLC has now been reconsidered. However, several groups have also reported that APP can associate with JIP, especially JIP1b (*16*, *49*, *50*). Moreover, JIP1, JIP2, and JIP3 bind KLC and this interaction mediates the association of the kinesin motor with transmembrane signaling molecules such as ApoER2 (*51*). The association of APP with KLC through JIP1b was confirmed, indicating that APP is able to serve as cargo in the presence of JIP1b. Membrane vesicles containing a Venus-APP fusion protein move on microtubule tracks in the presence of JIP1b (Y. Araki, M. Kinjyo and T. Suzuki., unpublished observation).

Second, the nerve ligation study was performed again, and the distributions of APP and PS1 in the axons were reexamined. APP accumulated at the region proximal to the ligature (*5*), but PS1 was distributed evenly through the axon (*12*). Furthermore, the kinesin-I motor-molecules were distributed throughout the axon independently of APP (*12*). These data seem to indicate that APP does not function as a cargo-receptor for kinesin-I. It is still unclear whether or not vesicles containing APP include PS and other secretases. However, it is important to note that KLC associates with APP through JIP1b, and JIP1b may be able to bind other cargo-receptor candidates. If so, the distribution of kinesin-I in axons may not be affected by the level of APP expression. Furthermore, it is not obvious whether a single vesicle carries only one type of cargo-receptor for a given motor molecule. A distinct membrane cargo-protein that binds to KLC directly and APP, which binds to KLC indirectly, may be included in a single vesicle, and KLC may select either receptor in the vesicle depending on the intracellular circumstances, such as the JIP1b gradient in the axon. Therefore, accumulating knowledge supports the ability of APP to function as a cargo-receptor of kinesin-I through the mediation of JIP1b, but further examination is needed to identify the content of the APP-cargo.

Vesicular transport deficits and Alzheimer's disease

Just after the isolation of APP as the precursor of Aβ in the late 1980s, many researchers found that the majority of APP is subjected to primary cleavage at the α-site, and only a limited amount of APP is cleaved at the β-site to generate Aβ. These early studies indicated that production of Aβ is a physiological and not a pathology-specific event in cells, and it is closely related to the protein secretory pathway. However, the question of where Aβ is generated in neurons has arisen. Although pathological disorders of axons such as terminal swelling and unusual axonal varicosities have been observed prior to amyloid deposition in APP-Tg mice, and a reduction in kinesin-I gene dosage tends to increase the generation of Aβ in mutant mice (*6*), whether axonal defects directly induce the facilitation of Aβ generation in axons is still being debated. Swelling of cholinergic axons has been detected in the brains of humans with early-stage AD prior to Aβ deposition, suggesting a relationship between deficient axonal transport and AD pathogenesis; we therefore expect that the molecular mechanisms and regulation of vesicular trafficking by AD-related molecules will continue to be studied.

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