Novel Cadherin-related Membrane Proteins, Alcadeins, Enhance the X11-like Protein-mediated Stabilization of Amyloid β-Protein Precursor Metabolism

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Previously we found that X11-like protein (X11L) associates with amyloid β-protein precursor (APP). X11L stabilizes APP metabolism and suppresses the secretion of the amyloid β-protein (Aβ) that are the pathogenic agents of Alzheimer’s disease (AD). Here we found that Alcadein (Alc), a novel membrane protein family that contains cadherin motifs and originally reported as cal- syntenins, also interacted with X11L. Alc was abundant in the brain and occurred in the same areas of the brain as X11L. X11L could simultaneously associate with APP and Alc, resulting in the formation of a tripartite complex in brain. The tripartite complex stabilized intracellular APP metabolism and enhanced the X11L-mediated suppression of Aβ secretion that is due to the retarda- tion of intracellular APP maturation. X11L and Alc also formed another complex with C99, a carboxy-terminal fragment of APP cleaved at the β-site (CTFβ). The formation of the Alc-X11L-C99 complex inhibited the inter- action of C99 with presenilin, which strongly sup- pressed the γ-cleavage of C99. In AD patient brains, Alc and APP were particularly colocalized in dystrophic neurites in senile plaques. Deficiencies in the X11L-me- diated interaction between Alc and APP and/or CTFβ enhanced the production of Aβ, which may be related to the development or progression of AD.

The production, aggregation, and accumulation of amyloid β-protein (Aβ)1 in the brain are initial steps in the pathogene-

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The abbreviations used are: Aβ, amyloid β-protein; Alc, Alcadein (Alzheimer’s disease-related cadherin-like protein); APP, amyloid β-protein precursor; AD, Alzheimer’s disease; NTF, amino-terminal fragment; CTF, carboxy-terminal fragment; CTFβ, carboxy-terminal fragment of APP cleaved at β-site; C99, CTFβ fragment expressed as CTFβ construct; CHAPS, 3-(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GM-130, 130-kDa Golgi matrix protein; GST, glutathione S-transferase; HA, hemagglutinin; KHC, kinesin heavy chain; PDZ, repeated sequences in the brain-specific protein PSD-95, the Drosophila septate junction protein disks-large, and the epithelial tight junction protein ZO-1; PS, presenilin; APP, large extracellular amino-terminal domain truncated at the α- and/or β-site; SYT, synaptotagmin; X11L, X11-like protein; h, human; XB31, X11L-binding protein clone number 31; SAD, sporadic AD; APPcyt, cytoplasmic domain of APP; PI, phosphatidylinositol interaction; HEK, human kidney embryonic; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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homologues of LIN-10 in Caenorhabditis elegans (18, 19) and dX11L in Drosophila melanogaster (20). They are neuron-specific adaptor proteins composed of a large amino-terminal region, a central phosphotyrosine interaction (PI) domain, and two carboxyl-terminal PDZ domains. The 681-GEYNPRTYG motif of APPct (numbering is based on the APP983 isoform) interacts with the PI domain of X11L (14).

To understand how APP metabolism is regulated, including Aβ generation, it is of interest to isolate other proteins that interact with X11L. This will help elucidate the molecular mechanism by which X11L regulates APP metabolism.

In the present study, we isolated novel human cDNAs encoding Alcadesins (Alcs), which are type I transmembrane proteins that interact with the PI domain of X11L. These genes are conserved in a wide variety of species, including D. melanogaster and C. elegans. We found that the cytoplasmic domain of Alc bound to the PI domain of X11L and initiated the formation of a tripartite complex comprised of Alc, X11L, and APP. This complex stabilized intracellular APP metabolism and significantly suppressed Aβ production by slowing APP maturation. We found that Alc and X11L also formed a tripartite complex with C99, a CTFβ. This complex inhibited the interaction of C99 with PS1, a component of γ-secretase that generates Aβ from C99. We could recover endogenous X11L-APP-Alc tripartite complexes from normal mouse brains, and normal mouse neurons displayed remarkable colocalization of these proteins.

In AD brains, APP was found to colocalize with Alc in the dystrophic neurites of senile plaques. These observations indicate that APP exists in protein complexes composed of X11L and Alc that regulate APP metabolism, including Aβ production in neurons, and that this regulatory mechanism may be perturbed in AD.

MATERIALS AND METHODS
cDNA Cloning of Human Alc1 and Plasmid Construction of Alca Family cDNAs—The yeast two-hybrid system used in this study has been described previously (14). Briefly, the human brain MATCHMAKER cDNA library (Clontech) was screened with bait composed of cDNA encoding part of the human X11L (hX11L) protein (amino acids 129–555). This yielded a cDNA clone (1X11L-binding protein clone number 21 (X3B1a)) that contained a partial open reading frame of Alc1. Using this clone, a clone encoding the full-length Alc1 protein was isolated from a human brain ag111 cDNA library (Clontech). This cDNA was designated as human Alc1 (GenBank™ accession number AF438492, registered as XB31a). The full-length hAlc1 open reading frame was subcloned into the HindIII and XbaI sites of pcDNA3 (Invitrogen). We identified two similar cDNA clones in the HUGE (Human Unidentified Gene-Encoded Large Proteins database) analyzed by the Unidentified Gene-Encoded Large Proteins database (accession number KIAA0726). We also found human Alc (accession number MM223141) in the GenBank™/EBI Data Bank. Alcβ cDNA, a generous gift from Dr. Nagase (KAZUSA DNA Research Institute, Chiba, Japan), was recloned into pcDNA3 at HindIII and XbaI sites to produce pcDNA3-hAlcβ. The human Alc1 and Alcβ cDNAs were also recloned via an XbaI site into pcDNA3 with a FLAG tag on the amino-terminal end so that pcDNA3-FLAG-hAlc1 and pcDNA3-FLAG-hAlcβ could be produced.

Protein Interaction Assays in Yeast—The MATCHMAKER two-hybrid system (Clontech) with pGBT9 and pGAD424 was used as described previously (22). β-Galactosidase activity in the yeast two-hybrid system was measured in a liquid assay using β-nitrophenyl galactopyranoside and was expressed in Miller units.

Antibodies—The anti-Alcα polyclonal rabbit antibody UT38 was raised against a peptide composed of Cys plus the sequence between positions 954 and 971 of human Alcα. The anti-Alcα polyclonal rabbit antibody was raised against a peptide composed of Cys plus the sequence between positions 954 and 968 of human Alcβ. The antihX11L polyclonal antibodies, UT29 and UT30, were obtained as described previously (14), and the anti-X11L monoclonal antibody mint2 was purchased from BD Biosciences. Anti-FLAG (M2, Sigma) and anti-HA (12CA5, Roche Diagnostics) monoclonal antibodies were purchased. The anti-APP cytoplasmic domain polyclonal antiserum, G369, has been described previously (23). The anti-Aβ-APP extracellular domain monoclonal (22C11, Roche Diagnostics, and LN27, Zymed Laboratories Inc.) and polyclonal (Sigma) antibodies were purchased. The 4G8 monoclonal anti-Aβ antibody raised against the Aβ (17–24) peptide was purchased from Signet Laboratories (Dedham, MA). All polyclonal antibodies except G369 were affinity-purified before use. Monoclonal antibodies specific for protein-disulfide isomerase (ID9, Stressgen Biotechnologies, Victoria, British Columbia, Canada), 130-kDa Golgi matrix protein (GM-130) (clone no. 35, BD Biosciences), synaptotagmin (SYT) (clone no. 41, BD Biosciences), mouse kinesin heavy chain (KHC) (H2, Chemicon International, Temecula, CA), PS1 carboxyl-terminal fragment (PS1-CTF; Chemicon International), and PS1 amino-terminal fragment (PS1-NTF; Chemicon International) were used.

Cloning of cDNAs—CO3T and human embryonic kidney 293 (HEK293) cells (~10⁶ cells) were transfected as described previously (14) with the indicated amounts of various combinations of the pcDNA3-hX11L, pcDNA3-hAlc1, pcDNA3-hAlcβ, pcDNA3APP695, and pcDNA3-PS1 plasmids. In another experiment, pcDNA3.1-HA-hX11, in which human X11 cDNA with an attached HA tag at the 5’-end was inserted into the NheI and EcoRV sites of pcDNA3.1, was transfected instead of pcDNA3-hX11L. pcDNA3-FLAG-hX11L and pcDNA3-FLAG-hX11, in which human X11L and X11 cDNA with an attached FLAG tag at the 5’-end, were also used in coimmunoprecipitation assay. Cells were harvested by lysis for 1 h on ice in CHAPS buffer, which consists of phosphate-buffered saline (PBS; 140 mM NaCl and 10 mM sodium phosphate (pH 7.4)) containing 10 mM CHAPS, 5 µg/ml chymostatin, 5 µg/ml leupeptin, and 10 µg/ml pepstatin (10) by strokes of a loose-fitting (0.12-mM) Teflon homogenizer. The postnuclear supernatants were further centrifuged at 100,000 × g for 1 h. The resulting precipitate (membrane fraction) was resuspended and separated by centrifugation for 115 min at 40,000 rpm in a Beckman SW41 rotor at 4 °C on a 0–28% (w/v) iodixanol density gradient. The supernatants were then fractionated into 13 tubes (0.8 ml/tube), and the fractions (20 µl) were analyzed by Western blot analysis with the indicated antibodies. Fraction 8 was solubilized by adding an equal amount of 2× CHAPS buffer consisting of 2× PBS (280 mM NaCl and 20 mM sodium phosphate (pH 7.4)) plus 20 mM CHAPS. SDS-PAGE was allowed to rotate for 30 min at 4 °C and then centrifuged at 100,000 × g for 60 min. The resulting gels used in the coimmunoprecipitation analysis of endogenous proteins.

Pulse-Chase Study—Pulse-chase labeling of cells was performed with [35S] in vitro Cell Labeling Mix (0.4 µCi/ml; AGQ 0082, Amer sham Biosciences). HEK293 cells were transfected with the indicated combinations of the pcDNA3APP695, pcDNA3-hX11L, pcDNA3-FLAG-hX11L, and pcDNA3 vector plasmids (transfection was performed with 9 µg of plasmids in total). After a 48-h transfection, the cells were metabolically labeled for 15 min. This was followed by a 0–8-h chase period, which was initiated by replacing the labeling medium containing excess amounts of unlabeled methionine. The cells were then lysed and subjected to immunoprecipitation with G369 (anti-APP), Alcα plasmid domain antibody (AlcαPeptide) or PS1 amino-terminal fragment (PS1-NTF) antibody (10) and affinity-purified antibodies except G369 were affinity-purified before use. Coimmunoprecipitation of γ-secretase subunits and APP was performed with 9 µg of plasmids in total). Cells were supplied with fresh growth medium 5 h after the start of transfection, and conditioned medium was collected 48 h after the medium replacement. Aβ40 and Aβ42 were quantified with sandwich ELISA by using
three types of Ab-specific monoclonal antibodies (27). Intracellular AP40 and AP42 were also extracted (28) and quantified by the sandwich ELISA. Briefly, the cells were lysed by sonication in 40 μl of PBS containing 6 mM guanidine chloride and centrifuged at 20,000 × g for 15 min at 4 °C. The resulting supernatant was diluted up to 12-fold by adding PBS and used in the ELISA. The single factor analysis of variance test followed by Tukey multiple comparisons was used to analyze differences among groups of data. Data were presented as means ± S.E.

Immunological Staining of Mouse Brain Sections—Experimental procedures were conducted in compliance with the guidelines of the Animal Studies Committee of Hokkaido University. Adult C57BL/6 mice (6 weeks, male) were perfused at 4 °C for 20 min with 4% (w/v) paraformaldehyde in 0.2 M sodium phosphate buffer, pH 7.5. The brains were excised and postfixed with the same fixative at 4 °C overnight followed by treatment with 30% (w/v) sucrose in PBS for 2–3 days at 4 °C. Brains were embedded into OCT compound (Miles Scientific, and frozen sagittal sections (20 μm) were prepared. The sections were incubated with 0.1% (v/v) Triton X-100 in PBS for 5 min at room temperature followed by treatment with 0.3% (v/v) H2O2 in PBS for 5 min at room temperature to quench endogenous peroxidase activity. The sections were blocked with 3% (w/v) bovine serum albumin in PBS containing 0.1% (v/v) Triton X-100 for 1 h at room temperature followed by incubation with 0.1% (v/v) H2O2 in PBS for 5 min at room temperature to quench endogenous peroxidase activity. The sections were blocked with 3% (w/v) bovine serum albumin in PBS for 10 min at room temperature and incubated for 3 h at 4 °C with antibodies in 1% (w/v) bovine serum albumin in PBS. After washing, the sections were further incubated with goat anti-mouse IgG coupled with Alexa Fluor 488 or goat anti-rabbit IgG coupled with Alexa Fluor 568 in 1% (w/v) bovine serum albumin for 1 h at room temperature. Sections were viewed using the confocal laser scanning microscope LSM510 (Carl Zeiss).

Immunological Staining of AD Brains—We used paraffin sections of frontal and temporal cortices from five AD subjects. Tissues were fixed with Kryofix (a mixture of ethanol, polyethylene glycol, and water; Merck) for 1–7 days and embedded in paraffin. Dewaxed serial tissue sections (cut into 4-μm sections) were immunostained with the ABC elite kit (Vector Laboratories, Burlingame, CA). Some sections were pretreated with a microwave antigen retrieval method for 10 min in 10 mM citrate buffer, pH 6.0 (29). Sections were incubated with antibodies specific for Alcα (UT83, 0.8 μg/ml), APP (22C11, 0.5 μg/ml), and Aβ (4G8, 1:1000 dilution). The peroxidase activity was visualized with diaminobenzidine-H2O2 solution. For control analyses, tissue sections were incubated with anti-Alcα antibody UT83 in the presence of the antigen peptide (40 nm) or non-immune rabbit IgG (0.8 μg/ml).

For double immunofluorescence analyses, dewaxed sections were incubated with a mixture of UT83 and 22C11 or of UT83 and 4G8 at the same dilutions as above followed by incubation with a mixture of fluorescein isothiocyanate-tagged goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:30 dilution) and Cy3-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 1:50 dilution). Prior to immunolabeling, the autofluorescence of the lipofuscin granules was blocked with Sudan black B staining (30).

RESULTS

Isolation, Identification, and Characterization of Novel Cadherin-related Membrane Protein Genes—Yeast two-hybrid screening of a human brain cDNA library with cDNA encoding a part of the hX11L protein resulted in the isolation of a novel cDNA denoted XB31a (GenBank accession number AF438482). The cDNA encoded a type I transmembrane protein composed of 971 amino acids. In this paper, this protein has been entitled human Alcadin (Alzheimer-related cadherin-like protein) α1 or Alcα (Fig. 1A). A thorough search of the human cDNA data base and genome data bases revealed three similar genes. These encode 981-, 968-, and 955-amino acid proteins, and we denoted these proteins as Alcα2, Alcβ, and Alcγ, respectively (Fig. 1A). Alcα2 is identical to human Alcα1 except for 10 additional amino acids between the Alcα1 residues at positions 71 and 72 (Fig. 1A, pink box). This extra sequence in Alcα2 is derived from one exon, and both proteins are spliced variants of the XB31a (Alcα) gene. The Alcβ and Alcγ proteins are both ~50% homologous to Alcα. They are encoded by different genes and belong to the same gene family to which Alcα belongs. Further data base examinations revealed that there are Alc-like genes in D. melanogaster (GenBank accession number AAF59384) and C. elegans (GenBank accession number NP495189) as well.

The Alc family members contain two cadherin motifs, a putative Ca2+-binding sequence in their amino-terminal halves, and a single cytoplasmic domain composed of ~110 amino acids (Fig. 1A). This cytoplasmic domain includes the X11L-binding site (see Fig. 2), which is highly conserved among different species, and an acidic region at the carboxyl-terminal end of the X11L-binding site (Fig. 1, A and B).

Expression and Distribution of Alcα—X11L is specifically expressed in neural tissue (14). We examined which human tissues express Alcα and Alcβ by Northern blot analysis, and we found strong expression of the ~5-kb Alcα transcript in the brain and weaker expression in the heart, while a probe specific for Alcβ revealed high levels of a 4.4-kb transcript in the brain (data not shown, please see Supplemental Fig. 1).

Expression of Alc protein in mouse brain tissue was examined by Western blot analysis. The anti-Alcα antibody UT83 recognizes two endogenous proteins in mouse brain lysates (Fig. 1C) as well as recombinant human Alcα1 but not recombinant Alcβ (data not shown, please see Supplemental Fig. 2). The antigen peptide to which UT83 was raised competed with the endogenous proteins for binding to UT83 (Fig. 1C, +). Thus, the UT83 antibody specifically recognizes two endogenous Alcα proteins in the mouse brain. When UT83 was used to analyze Alcα expression in a variety of murine tissues, the highest levels were found in the brain, and small amounts were found in the lung (Fig. 1D, upper panel). Assessment of X11L expression with the UT30 X11L-specific antibody confirmed its brain-specific distribution (Fig. 1D, upper panel). These observations suggest that, like X11L, Alcα is abundant in neurons. This colocalization in the brain supports the notion that X11L and Alcα interact physiologically. This idea was further supported when we examined Alcα and X11L expression in various parts of the brain by dissecting adult mouse brains into various component regions and blotting them with UT83 and UT30. Relatively high levels of Alcα were detected in the cerebral cortex, striatum, hippocampus, and thalamus; moderate levels were found in the cerebellum; and low levels were observed in the olfactory bulb, midbrain, and pons (Fig. 1D, lower panel).

Expression in the sciatic nerve fiber was not observed. X11L was found in the cerebellum; and low levels were observed in the olfactory bulb, midbrain, and pons (Fig. 1D, lower panel). Expression in the sciatic nerve fiber was not observed. X11L was distributed in a very similar pattern (Fig. 1D, lower panel). Thus, X11L and Alcα are both largely expressed in neural tissues apart from peripheral nervous system.

Alc and X11L Interaction—We investigated the interactions between Alc and X11L by coimmunoprecipitation assays from COS7 cells expressing X11L together with Alcα1 or Alcβ followed by Western blot analysis. The immunoprecipitates resulting from incubating the lysates with anti-Alcα (UT83) or anti-Alcβ (BS7) antibodies included X11L as detected by the X11L antibody (UT30) (data not shown, please see Supplemental Fig. 3). Conversely the immunoprecipitates generated by the anti-X11L antibody contained Alcα1 and Alcβ. In contrast, non-immune antibody (control IgG) immunoprecipitates did not contain these proteins. Thus, both the Alcα and Alcβ proteins bind to X11L within cells.

We identified the Alcα- and Alcβ-binding domain of X11L by assessing the in vitro binding of various X11L domain constructs to the putative cytoplasmic domains of Alcα and Alcβ. Cell lysates containing X11L protein constructs were incubated with beads coupled to the cytoplasmic domains of human Alcα or Alcβ fused to GST. GST alone was used as a negative control. The pull-downs were subjected to Western blot analysis with the anti-X11L amino-terminal antibody UT29. Full-length X11L and its construct encoding the X11L amino-terminal domain attached to the PI domain (N + PI) bound the GST-Alcα
indicated as an acidic region. The plasma membrane is a conserved region containing the X11L-binding sequence. The pink box in the hAlcα sequence indicates the amino acids derived from an exon of the hAlcα gene. Yellow boxes include the predicted cadherin motif, while the black box within the human Alcβ sequence represents the leucine zipper motif. The green box includes a highly conserved region containing the X11L-binding sequence. The red box indicates an acidic region. The plasma membrane is indicated as a purple ladder-back structure. The underlines indicate the regions recognized by the specific antibodies UT83 and BS7. The numbers indicate the amino acid (a.a.) residues. B, amino acid sequences of the cytoplasmic domains of Alc family proteins. The arrow reveals the NP motif that is the X11L-binding site (blue). Gaps produced by the alignment are indicated by a hyphen in the sequence. The numbers indicate amino acid position. C, detection of Alc proteins in the mouse brain by the UT83 Alc antibody. Mouse brain lysates (50 μg of protein) were subjected to SDS-PAGE (6% (w/v) polyacrylamide), transferred to a membrane, and probed with UT83 (0.16 μg/ml IgG) in the presence (+) or absence (−) of the antigen peptide used to raise it (40 nm). UT83 specifically detected two Alcα proteins. The numbers refer to the molecular masses (kDa) of the protein standards. D, analysis of the distribution of Alcα and X11L in various murine tissues and brain regions by Western blot analysis. Mouse lysates (50 μg of protein) of tissues (upper panel) and brain regions (lower panel) were subjected to SDS-PAGE (6% (w/v) polyacrylamide), transferred to the membrane, and probed with the anti-Alc (UT83) and anti-X11L (UT90) antibodies. Br, brain; Ht, heart; Lu, lung; Li, liver; Kid, kidney; Mus, muscle; OB, olfactory bulb, CC, cerebral cortex; ST, striatum; Hip, hippocampus; Ce, cerebellum; Mid, mid brain; Th, thalamus; Sci, sciatic nerve.

FIG. 1. Comparison of structures and cytoplasmic amino acid sequences of the Alc family proteins and protein expression of Alcα and X11L. A, a schematic representation of structural features of the human Alc proteins. The pink box in the hAlcα sequence indicates the amino acids derived from an exon of the hAlcα gene. Yellow boxes include the predicted cadherin motif, while the black box within the human Alcβ sequence represents the leucine zipper motif. The green box includes a highly conserved region containing the X11L-binding sequence. The red box indicates an acidic region. The plasma membrane is indicated as a purple ladder-back structure. The underlines indicate the regions recognized by the specific antibodies UT83 and BS7. The numbers indicate amino acid (a.a.) residues. B, amino acid sequences of the cytoplasmic domains of Alc family proteins. The arrow reveals the NP motif that is the X11L-binding site (blue). Gaps produced by the alignment are indicated by a hyphen in the sequence. The numbers indicate amino acid position. C, detection of Alc proteins in the mouse brain by the UT83 Alc antibody. Mouse brain lysates (50 μg of protein) were subjected to SDS-PAGE (6% (w/v) polyacrylamide), transferred to a membrane, and probed with UT83 (0.16 μg/ml IgG) in the presence (+) or absence (−) of the antigen peptide used to raise it (40 nm). UT83 specifically detected two Alcα proteins. The numbers refer to the molecular masses (kDa) of the protein standards. D, analysis of the distribution of Alcα and X11L in various murine tissues and brain regions by Western blot analysis. Mouse lysates (50 μg of protein) of tissues (upper panel) and brain regions (lower panel) were subjected to SDS-PAGE (6% (w/v) polyacrylamide), transferred to the membrane, and probed with the anti-Alc (UT83) and anti-X11L (UT90) antibodies. Br, brain; Ht, heart; Lu, lung; Li, liver; Kid, kidney; Mus, muscle; OB, olfactory bulb, CC, cerebral cortex; ST, striatum; Hip, hippocampus; Ce, cerebellum; Mid, mid brain; Th, thalamus; Sci, sciatic nerve.

and GST-Alcβ fusion proteins (data not shown, please see Supplemental Fig. 4). However, the amino-terminal domain alone did not bind either protein. GST alone also did not bind to any of the X11L constructs. Thus, as with APP (14), the cytoplasmic domains of Alcα and Alcβ bind the PI domain of X11L.

We also identified the region in the cytoplasmic domain of Alcα (Alcα-CTF) that is required for the interaction with X11L by yeast two-hybrid analysis. Truncated Alcα1CTF constructs lacking the amino-terminal regions (CTF1, Δ871–902; CTF2, Δ871–908) or carboxy-terminal regions (CTF3, A903–971; CTF4, A909–971) were examined for interaction with the N + PI domain (amino acids 129–555) of X11L. β-Galactosidase activity was measured in a liquid assay and calculated as Miller units. The CTF1 and CTF4 constructs, which have the conserved 904 NPMETY909 (numbering is based on the hAlcα isoform) sequence in common, bound X11L, but CTF2 and CTF3, which both lack the NPMETY sequence, did not interact with X11L (Fig. 2). The NPXXXY sequence is a modified version of the NPXY motif of the cytoplasmic domain of APP that binds X11L (14). When we introduced mutations into the first two amino acids of the NPMETY sequence generating AMETY, denoted as NP-AA, AlcαCTF did not interact with X11L. However, modification of NPMETY to NPMETA (Y-A) had no effect on the ability of the protein to bind to X11L (Fig. 2). Thus, it appears that the first two amino acids of the NPMETY motif are essential for the association of Alcα with X11L. This is supported by the mutational analyses of the site used by APP to bind X11 as it was shown that the end tyrosine residue of the NPTY motif of APP is not important for binding to X11L (31). Moreover, the first two Asp and Pro amino acids are conserved in all the Alc proteins, whereas the end Tyr residue is not conserved in Alcβ or the D. melanogaster and C. elegans Alc proteins (Fig. 1B).

Alc, X11α, and APP Form a Tripartite Complex—The cytoplasmic domains of Alc and APP both bind to the PI domain of X11L. We thus investigated whether Alc and APP compete for binding to X11L or act cooperatively. APP695 (the human APP isoform composed of 695 amino acids) and X11L were expressed together in HEK293 cells in the presence or absence of hAlcα or FLAG-tagged hAlcβ, and APP was immunoprecipitated from cell lysates with the anti-APP cytoplasmic domain antibody G369. The immunoprecipitates were analyzed by Western blot
Suppression of Aβ by X11L and Alc aden association with APP

Fig. 2. Determination of the X11L-binding site of Alc aden. Various protein constructs based on the cytoplasmic domain of Alc aden (shown schematically in the upper panel) were expressed in yeast and examined by the yeast two-hybrid system for their ability to bind X11L. The first 125 amino acids in the amino-terminal domain of X11L were deleted as they generate a nontoxic and constitutive positive signal in this assay system (14). The following Alc aden deletion and point mutants were tested: wild type (wt), the cytoplasmic domain of Alc aden (amino acids 871–971 of Alc aden 1); CTF1, amino acids 903–971; CTF2, amino acids 909–971; CTF3, amino acids 871–902; CTF4, amino acids 871–908; NP-α, Alc aden substitution for Asn-Pro in NPMETY sequence; Y-A, Alc aden substitution for Tyr in NPMETY. The ability to grow in selective medium was examined, and β-galactosidase activity was quantified by a liquid assay and calculated in Miller units ± S.D. (lower panel, n = 3). The plasmid alone was used as a control. GAL4AD represents the yeast Gal4 activator domain.

analysis with anti-APP (G369), anti-X11L (UT29), anti-Alc aden (UT33), and anti-FLAG (M2) antibodies. As reported previously (14), X11L was coimmunoprecipitated with the anti-APP antibody in the absence of Alc aden. Surprisingly, in the presence of Alc aden 1 or FLAG-Alc β, higher levels of X11L were recovered in the APP immunoprecipitates (data not shown). However, the majority of X11L was recovered along with X11 aden in the cytoplasmic fraction, although a moderate proportion was recovered in the membrane fraction (data not shown). However, the majority of X11L was recovered in the cytoplasmic fraction, although a moderate amount of X11L was also recovered in the membrane fraction (data not shown). To examine formation of these proteins into tripartite complexes in the brain, the membrane fraction was further fractionated by iodixanol density gradient centrifugation, and these fractions were subjected to Western blot analysis with antibodies specific for APP, X11L, Alc aden, the endoplasmic reticulum protein protein-disulfide isomerase, the Golgi-resident protein GM-130, the synaptic vesicle protein SYT, the vesicle transport motor protein KHC, and PS1. The vesicles bearing APP, APPCTF, X11L, and Alc aden all sedimented in the same medium density membranous protein fractions 7–9, which were a bit heavier than the SYT-containing vesicles in fractions 9 and 10 (Fig. 4A). APP, KHC, and PS1 were observed in the same fractions, confirming earlier observations (33, 34). These data suggest that APP, X11L, and Alc aden could form tripartite complexes on organelles composed of medium density membranes. To test this, the proteins in fraction 8 were solubilized with CHAPS buffer, immunoprecipitated with anti-APP (G369) or non-immune control antibodies, and subjected to Western blot analysis with antibodies specific for APP, X11L, Alc aden, SYT, and rabbit IgG antibodies. Alc aden and X11L were coimmunoprecipitated by anti-APP antibody but not by non-immune antibody (Fig. 4B). Also SYT was not coimmunoprecipitated by anti-APP antibody. Thus, APP and/or APPCTF, Alc aden, and X11L form a tripartite complex in vivo, probably on medium density membrane organelles that contain cargo proteins including PS1 and that differ from the synaptic vesicles that contain SYT.

We next investigated whether APP, X11L, and Alc aden colocalize in neurons. Sagittal sections of the hippocampus from adult mouse brains were double stained with antibodies specific for

2 Y. Araki and T. Suzuki, unpublished observation.
3 A. Sumioka and T. Suzuki, unpublished observation.
Suppression of Aβ by X11L and Alcadein Association with APP

Fig. 3. Alc enhances APP-X11L binding, and the three proteins form a tripartite complex. A, role of Alc in the binding of X11L to APP. HEK293 cells (2 x 10⁶ cells) were transiently cotransfected with pcDNA3APP695 (2 µg) and pcDNA3-hX11L (0.25 µg) with or without pcDNA3-FLAG-hAlc1 (6.75 µg) or pcDNA3FLAG-hAlcβ (6.75 µg) as indicated. To standardize the plasmid concentrations, the pcDNA3 vector (−) was added (to yield 9 µg of plasmid in total). The cells were lysed, and APP in the cell lysate was recovered by immunoprecipitation with the anti-APP cytoplasmic domain antibody G369. The immunoprecipitates were then analyzed by Western blotting with anti-APP (G369), anti-X11L (UT29), anti-Alc1 (UT83), and anti-FLAG (M2) antibodies. B, role of X11L in the formation of the APPX11L:Alc tripartite complex. HEK293 cells (2 x 10⁶ cells) were transiently cotransfected with pcDNA3APP695 (2 µg) and pcDNA3-FLAG-hAlc (1.0) or pcDNA3-FLAG-hAlcβ (0.25 µg) in various combinations. To test this, we assessed the intracellular APP metabolism in HEK293 cells that express APP with or without X11L in the presence or absence of Alc1 by pulse-chase assay (Fig. 5). The metabolically radiolabeled APP was recovered by immunoprecipitation at the indicated chase periods and separated by SDS-PAGE. The levels of immature APP were quantified by autoradiography and calculated with respect to the 0 h levels (1.0) (Fig. 5B). When APP was expressed alone, the immature APP levels decreased gradually with time due to the maturation of APP and the secretion of large extracellular domain truncated at α- or β-site (sAPP) during the chase period. As expected, X11L coexpression slightly delayed this decrease in immature APP levels, indicating that X11L stabilizes APP metabolism. When Alc1 was also coexpressed, the effect of X11L was greatly enhanced. However, the increased stabilization of APP metabolism due to Alc1 was not observed if X11L was not coexpressed.

We next investigated the production of Aβ and sAPP in Neuro-2a cells expressing APP with or without X11L in the presence or absence of Alc1. The amount of Aβ in medium secreted from the cells was quantified using sandwich ELISA. As we previously demonstrated (14), coexpression of X11L suppresses the secretion of Aβ40 (Fig. 5C). X11L did tend to suppress Aβ42 secretion, but it was not significant statistically. When X11L and Alc1 were coexpressed, the effect of X11L was remarkably enhanced. However, enhanced suppression of Aβ40 secretion was not observed if only Alc1 was expressed. Alc1 and X11L coexpression did not significantly affect Aβ42 secretion. Thus, Alc clearly enhances the X11L-mediated inhibition of Aβ and Alc1 and X11L, or APP and Alc1. The three proteins largely colocalized (Fig. 4C). Strong X11L immunoreactivity was detected in the CA3 pyramidal cell somata and proximal dendrites where APP and Alcα were also localized (Fig. 4C). Colocalization was observed in the large pyramidal neurons of the cerebral cortex (data not shown). High power views of the cell bodies of these neurons confirmed the colocalization of APP, Alcα, and X11L (Fig. 4C). Thus, neurons expressing X11L also expressed Alcα and APP, and the three proteins colocalize in these cells.

Alc Enhances the X11L-mediated Stabilization of APP Metabolism and Suppression of Aβ and sAPP Secretion—X11 stabilizes intracellular APP metabolism (35, 36), and we previously reported that X11L suppresses the secretion of Aβ40 (14), indicating that X11L also stabilizes APP metabolism. Since Alc and APP form a complex with X11L through cytoplasmic interactions, and this complex formation strengthens the interaction between APP and X11L (Fig. 3), we speculated that Alc may enhance the X11L-mediated stabilization of APP metabolism and thereby further suppress Aβ and sAPP production. To test this, we assessed the intracellular APP metabolism in HEK293 cells that express APP with or without X11L in the presence or absence of Alc1 by pulse-chase assay (Fig. 5). The metabolically radiolabeled APP was recovered by immunoprecipitation at the indicated chase periods and separated by SDS-PAGE. The levels of immature APP were quantified by autoradiography and calculated with respect to the 0 h levels (1.0) (Fig. 5B). When APP was expressed alone, the immature APP levels decreased gradually with time due to the maturation of APP and the secretion of large extracellular domain truncated at α- or β-site (sAPP) during the chase period. As expected, X11L coexpression slightly delayed this decrease in immature APP levels, indicating that X11L stabilizes APP metabolism. When Alc1 was also coexpressed, the effect of X11L was greatly enhanced. However, the increased stabilization of APP metabolism due to Alc1 was not observed if X11L was not coexpressed.

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bition of Aβ, at least Aβ40, secretion from APP, which corre-
lates with the finding that Alc stabilizes the interaction be-
tween X11L and APP (Fig. 3A). The amount of sAPP in the
medium secreted from the cells was detected by Western blot
analysis with anti-APP extracellular domain antibody 22C11
(Fig. 5D). X11L suppressed sAPP secretion, and Alc enhanced this effect. It is likely that the decreased Aβ40 and
sAPP secretion by cells expressing APP, X11L, and Alc is due to
the slow-down of intracellular APP maturation caused by their
tripartite complex formation. Because we could not quantify
the Aβ levels in the cell lysates, we performed another study
with cells expressing C99 protein (see Fig. 6).
X11L and Alc Form a Tripartite Complex with C99 and Suppress the γ-Cleavage of C99—As we have shown in Fig. 4, APP not only colocalized with X11L and Alc in the brain, the tripartite complex also colocalized with PS1. This suggests that X11L and Alc could suppress the γ-cleavage of the CTF of APP. We investigated this possibility by cotransfecting Neuro-2a cells with C99 instead of full-length APP with or without X11L and in the presence or absence of Alc1. The stability of C99 was examined by Western blot analysis, which revealed that X11L coexpression caused the intracellular C99 to accumulate (Fig. 6A). This suggests that X11L protects the CTF from γ-cleavage. This effect was enhanced when Alc1 was also coexpressed (Fig. 6A). However, the stabilization of intracellular C99 was not observed if Alcα was coexpressed in the absence of X11L.

When the Aβ in the medium from the transfected Neuro-2a cells described above was quantified, it was found that X11L coexpression with C99 suppresses the secretion of both Aβ40 and Aβ42 into medium (Fig. 6B). Moreover, when Alc1 was also coexpressed, this effect was remarkably enhanced (Fig. 6B). We could not quantitate the level of intracellular Aβ derived from full-length APP. Thus, we quantified the Aβ in cells expressing C99. When the intracellular levels of Aβ in the transfected Neuro-2a cells were examined, the same effects of X11L and Alc1 expression were observed for intracellular Aβ40 generation (Fig. 6C). However, expression of X11L and Alc1 did not have a significant effect on the intracellular Aβ42 levels, although the expression of both X11L and Alc1 did tend to suppress the amount of intracellular Aβ42 (Fig. 6C). Thus, we concluded that X11L could suppress the γ-cleavage of CTFβ, and Alc enhances this effect.

PS is essential for γ-secretase activity (37). We investigated whether X11L inhibits the interaction of CTFβ with PS1 and whether Alc enhances this inhibitory activity. HEK293 cells were cotransfected with C99 and PS1 in the presence or absence of X11L and with or without Alc1. These cells were subjected to immunoprecipitation assays with the anti-APP cytoplasmic domain antibody G369, separated by SDS-PAGE (6% (w/v) polyacrylamide), detected by autoradiography (A), and quantified using a Fuji BAS 2000 analyzer. The relative ratios of the levels of immature APP to the maximum level of immature APP at 0 h (the latter level was assigned a reference value of 1.0) were calculated (B). mAPP, mature APPPL, immature APPPL, C, effect of coexpressing Alc1 and X11L on Aβ secretion. Neuro-2a cells (~1 × 10⁶ cells) were transiently transfected with pCDNA3APPPL695 (2 μg) in the presence or absence of pCDNA3-hX11L (0.25 μg) and with or without pCDNA3FLAG-hAlc1 (0.75 μg). To standardize the plasmid amounts, pCDNA3 vector was added (to yield 9 μg of plasmid in total). After a 48-h transfection, the cells were metabolically pulse-labeled with [35S]methionine for 15 min and chased for the indicated times. After a 48-h transfection, the cells were metabolically pulse-labeled with [35S]methionine for 15 min and chased for the indicated times. APP was immunoprecipitated from cell lysates with the anti-APP cytoplasmic domain antibody G369, separated by SDS-PAGE (6% (w/v) polyacrylamide), detected by autoradiography (A), and quantified using a Fuji BAS 2000 analyzer. The relative ratios of the levels of immature APP to the maximum level of immature APP at 0 h (the latter level was assigned a reference value of 1.0) were calculated (B). mAPP, mature APPPL, immature APPPL, C, effect of coexpressing Alc1 and X11L on Aβ secretion. Neuro-2a cells (~1 × 10⁶ cells) were transiently transfected with pCDNA3APPPL695 (3 μg) with or without pCDNA3-hX11L (0.3 μg) and in the presence or absence of pCDNA3-FLAG-hAlc1 (5.7 μg). To standardize the plasmid amounts, pCDNA3 vector was added (to yield 9 μg of plasmid in total). The culture medium was collected and assessed for Aβ40 and Aβ42 levels using a sandwich ELISA. The concentrations of Aβ40 and Aβ42 are presented as means with S.E. (n = 6). The data were analyzed by one-way analysis of variance followed by the Tukey test (**, p < 0.01; ***, p < 0.001). D, effect of coexpressing Alc1 and X11L on Aβ secretion. Neuro-2a cells (~1 × 10⁶ cells) were transiently transfected with pCDNA3APPPL695 (3 μg) with or without pCDNA3-hX11L (0.3 μg) and in the presence or absence of pCDNA3-FLAG-hAlc1 (5.7 μg). To standardize the plasmid amounts, pCDNA3 vector was added (to yield 9 μg of plasmid in total). The culture medium (2 ml) was collected, and sAPP was recovered by immunoprecipitation with anti-APP extracellular domain antibody 22C11 as described previously (4). The immunoprecipitates (medium) and cell lysates (Cell, ~50 μg of protein) were analyzed by Western blotting with 22C11. The levels of sAPP (a relative ratio) were normalized to the amount of intracellular full-length mature and immature APP (APPm) and indicated relative to the level of lane 1, which was assigned a reference value of 1.0. Results are the average of duplicate assays, and error bars are indicated.

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Fig. 5. Alcα enhances the X11L-mediated stabilization of APP metabolism and suppression of Aβ and sAPP secretion. A and B, effect of coexpressing Alc1 and X11L on APP metabolism. HEK293 cells (~1 × 10⁷ cells) were transiently transfected with pCDNA3APPPL695 (2 μg) in the presence or absence of pCDNA3-hX11L (0.25 μg) and with or without pCDNA3FLAG-hAlc1 (0.75 μg). To standardize the plasmid amounts, pCDNA3 vector was added (to yield 9 μg of plasmid in total). After a 48-h transfection, the cells were metabolically pulse-labeled with [35S]methionine for 15 min and chased for the indicated times. APP was immunoprecipitated from cell lysates with the anti-APP cytoplasmic domain antibody G369, separated by SDS-PAGE (6% (w/v) polyacrylamide), detected by autoradiography (A), and quantified using a Fuji BAS 2000 analyzer. The relative ratios of the levels of immature APP to the maximum level of immature APP at 0 h (the latter level was assigned a reference value of 1.0) were calculated (B). mAPP, mature APPPL, immature APPPL, C, effect of coexpressing Alc1 and X11L on Aβ secretion. Neuro-2a cells (~1 × 10⁶ cells) were transiently transfected with pCDNA3APPPL695 (3 μg) with or without pCDNA3-hX11L (0.3 μg) and in the presence or absence of pCDNA3-FLAG-hAlc1 (5.7 μg). To standardize the plasmid amounts, pCDNA3 vector was added (to yield 9 μg of plasmid in total). The culture medium was collected and assessed for Aβ40 and Aβ42 levels using a sandwich ELISA. The concentrations of Aβ40 and Aβ42 are presented as means with S.E. (n = 6). The data were analyzed by one-way analysis of variance followed by the Tukey test (**, p < 0.01; ***, p < 0.001). D, effect of coexpressing Alc1 and X11L on Aβ secretion. Neuro-2a cells (~1 × 10⁶ cells) were transiently transfected with pCDNA3APPPL695 (3 μg) with or without pCDNA3-hX11L (0.3 μg) and in the presence or absence of pCDNA3-FLAG-hAlc1 (5.7 μg). To standardize the plasmid amounts, pCDNA3 vector was added (to yield 9 μg of plasmid in total). The culture medium (2 ml) was collected, and sAPP was recovered by immunoprecipitation with anti-APP extracellular domain antibody 22C11 as described previously (4). The immunoprecipitates (medium) and cell lysates (Cell, ~50 μg of protein) were analyzed by Western blotting with 22C11. The levels of sAPP (a relative ratio) were normalized to the amount of intracellular full-length mature and immature APP (APPm) and indicated relative to the level of lane 1, which was assigned a reference value of 1.0. Results are the average of duplicate assays, and error bars are indicated.
The localization of Alc/H9251 and APP, paraffin-embedded tissue sections from AD brains were double labeled for Alc/H9251 and APP (Fig. 7, panels 4–6). In a high power view of a neuritic plaque, most of the Alc/H9251 immunofluorescence (panel 4) colocalized with that of APP (panel 5). The merged view is shown in panel 6. Furthermore double labeling of Alc/H9251 and A/H9252 revealed Alc/H9251-positive neurites around the amyloid core of plaques (Fig. 7, panel 7). Unfortunately antibody specific for X11L (mint2) did not work on the paraffin-embedded tissue sections (data not shown). These observations suggest that in AD, Alc and APP accumulate in dystrophic neurites around the amyloid core of plaques.

**DISCUSSION**

The production, secretion, and aggregation of Aβ may cause neural cell death, resulting in the onset of AD. However, the cellular mechanisms involved in this neural cell death remain to be elucidated (2). The mechanisms involved in the development of familial AD involve mutations in APP and PS that appear to increase Aβ production and cause the early onset of AD. 

The Alc/H9251/X11L-C99 tripartite complex inhibits Aβ generation from C99 and blocks the association between C99 and PS1. A, effect of X11L and Alc on C99 cleavage. Neuro-2a cells (~1 x 10⁷ cells) were transiently transfected with pcDNA3-APPC99 (3 µg) in the presence or absence of pcDNA3-hX11L (0.3 µg) and with and without pcDNA3-hAlc1 (5.7 µg). To standardize the plasmid concentrations, pcDNA3 vector (–) was added (to yield 9 µg of plasmid in total). The cells were lysed, and C99 was analyzed by Western blotting with anti-APP (G369) antibody. The lower panel indicates a shorter exposure (exp) of the film. B and C, effect of X11L and Alc on the generation of Aβ from C99. Aβ40 (left panel) and Aβ42 (right panel) in the medium (B) and the lyse (C) of the cells in A were quantified by a sandwich ELISA. The Aβ40 and Aβ42 concentrations are presented as means with S.E. (n = 6). The data were analyzed by one-way analysis of variance followed by the Tukey test (*, p < 0.05; **, p < 0.01; ***, p < 0.001). D, effect of X11L and Alc1 on the interaction between C99 and PS1. HEK293 cells (~1 x 10⁶ cells) were transiently transfected with pcDNA3-APPC99 (2 µg) and pcDNA3-PS1 (1 µg) in the presence or absence of pcDNA3-hX11L (2 µg) and with or without pcDNA3-hAlc1 (4 µg). To standardize the plasmid amounts, pcDNA3 vector (–) was added (to yield 9 µg of plasmid in total). The cells were cultured for 24 h in the presence (+) or absence (–) of N-acetyl-leucyl-norleucinal (LLnL) (10 µM) and then lysed. C99 in the cell lysate was recovered by immunoprecipitation with the G369 anti-APP antibody. The immunoprecipitates were analyzed by Western blotting with antibodies specific for APP (G369), the full-length PS1 (PS1-NTF), X11L (mint2), and Alc1 (UT83). E, schematic diagram showing how the tripartite complex composed of CTFβ, X11s (X11L and X11), and Alc can block the γ-cleavage of CTFβ by PS. EC1 and EC2 in Alcadein indicate cadherin motifs 1 and 2, respectively. Although the stoichiometry in the complex APP-X11L/X11-Alc is drawn as 1:1:1 for the sake of convenience, further analysis is needed to reveal the substance of the complex.
Suppression of Aβ by X11L and Alcdein Association with APP

Supporting the notion that the Alc proteins participate in neural function(s) is that a chicken protein that is homologous to Alc has been reported recently to be a postsynaptic membrane protein that may play a role in postsynaptic Ca$^{2+}$ signaling (47). It is possible that Alc may transmit unidentified extracellular information through an as yet unknown mechanism or that it may serve as a receptor, together with APP, of cargo proteins in membrane transport vesicles (39). Supporting the first possibility, we demonstrated in the present study that Alc couples with APP through cytoplasmic interactions bridged by X11L or X11. APPcet is thought to transmit some extracellular signals into nucleus by the mechanism of regulated intracellular proteolysis by coupling with adaptor proteins such as FE65 (48). Alc and X11L may moderate this signal processing by regulating APP processing by γ-secretase complex including PS. Supporting the possible cargo protein receptor function of Alc is our demonstration that APP, X11L, and Alc were recovered together with KHC in identical subcellular fractions. However, there is no direct evidence that APP and Alc operate as receptors of cargo proteins in membrane transport vesicles (39).

We found that the coupling of APP with Alc through X11L significantly stabilized APP metabolism and enhanced the suppression of Aβ production. This effect was due to the suppression of APP maturation, resulting in the suppression of the first cleavage of APP at the α- and β-sites. Normally the majority of APP is subjected to non-amyloidogenic processing by α- and γ-secretases that does not generate Aβ (2), although a small proportion of APP is processed into Aβ by an intracellular amyloidogenic pathway in which the protein is cleaved by β- and γ-secretases. We found that X11L could also associate with the carboxyl-terminal fragments of APP that are metabolic products of APP cleavage at the α- or β-site. Thus, we examined whether X11L and Alc could also suppress the γ-cleavage of C99/CTFβ. We found that the X11L-mediated association of Alc with C99 enhanced the suppressive effects of X11L on Aβ generation from C99. Moreover, since a recent report indicates that PS is an essential component of γ-secretase (35), we examined whether the interaction between C99/CTFβ and PS1 is inhibited by formation of the C99-X11L-Alc complex. We found that X11L blocked PS from interacting with C99/CTFβ and that Alc enhanced this effect. The stable tripartite complex formed by C99/CTFβ, X11L, and Alc may block the access of the γ-secretase complex to CTFβ. These observations suggest that the development of drugs that up-regulate X11L and Alc function in AD patients and thereby down-regulate CTFγ cleavage may be useful in the treatment of AD.

Both APP and Alc recognized the PI domain of X11L, but the binding of these two proteins to PI was cooperative, not competitive. The association of Alc with X11L may induce some conformational change of the PI domain of X11L and result in the stable interaction of APP with X11L. The detailed analysis of the mechanism for interaction among three proteins, including the determination of the stoichiometry of proteins in the complex, is under consideration.

We found that the NPXXX motif in the cytoplasmic domain of Alc was responsible for the interaction between Alc and the PI domain of X11L. The first and second residues (Asn and Pro) were essential for these interactions, unlike the end Tyr residue. Supporting this is that the Tyr residue is not conserved in all of the Alc family molecules. Moreover the NPXY motif in APP, which is responsible for the interaction between APP and X11L, does not require conservation of the end Tyr residue (31). Thus, while the Tyr residue may be important, perhaps as a possible phosphorylation site, it is not required for interaction with X11L.
APP immunolabeling is a sensitive method for detecting disturbances in axonal transport (49, 50) and consistently identifies dystrophic neurites in the senile plaques in both non-demented and AD brains (51, 52). In this study, we observed that Alcα accumulated in the plaque neurites along with APP. However, X11L, Alc, and APP were found to colocalize largely in normal murine neurons. It is likely that deficient interactions between Alc and APP, by implying axonal transport, generate greater amounts of APPβ in dystrophic neurons of AD patients.

Our studies and those of others reveal that various cytoplasmic and membrane proteins such as X11L and Alc interact with APPcyt to control APPα production. This information may prove highly useful for the development of novel therapeutic drugs that suppress APPβ production in SAD cases.

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Addendum—Alcadin is a molecule identical to calyxtenin, which has been published by Hintsch et al. (54).
Novel Cadherin-related Membrane Proteins, Alcadeins, Enhance the X11-like Protein-mediated Stabilization of Amyloid β-Protein Precursor Metabolism
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