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Synergistic Effects of Munc18a and X11 Proteins on Amyloid Precursor Protein Metabolism*

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X11 proteins have been shown to modulate metabolism of the amyloid precursor protein (APP) and to reduce the secretion of β-amyloid peptides (Aβ) that are associated with Alzheimer's disease. Whereas X11α interacts with APP via its phosphotyrosine-binding domain, recent reports indicate that additional regulatory interactions involve the N terminus of X11. Here we report that the syntaxin-1a-binding protein Munc18a, which interacts with the Munc18a-interacting domain (MID) at the N terminus of X11, strongly regulates the actions of X11 on APP metabolism. When co-expressed with X11α, Munc18a potentiated the retention of APP and suppression of Aβ secretion by X11α. As a result, the constitutive release of Aβ40 was nearly abolished. Experiments using N terminus deletion mutants of X11α/β and the MID-deficient X11γ revealed that the majority of the regulatory effect by Munc18a occurred independent of a direct interaction of Munc18a with X11, although the presence of X11 was required. Munc18a expression induced a small increase in β-secretase activity, whereas it also intensified the reduction in Aβ40 secretion by X11α. These data indicate that Munc18a in concert with X11 acts to suppress γ-secretase processing. We conclude that Munc18a acts through direct and indirect interactions with X11 proteins and powerfully regulates APP metabolism and Aβ secretion.

The two major pathological features in the brains of patients with Alzheimer's disease are the presence of β-amyloid peptide (β-AP or Aβ)[1] containing senile plaques and neurofibrillary tangles (1). Aβ peptides were derived from proteolytic processing of a precursor protein, namely amyloid precursor protein (APP). The cleavage sites and extent of processing depend on its trafficking pathways as different APP derivatives have been mapped to distinct compartments of the cell, presumably where specific APP processing enzymes, or secretases, reside (2–6). A major pathway for Aβ production involves internalization of APP, as directed by an ENPTY sequence at the C terminus of APP, following its delivery to the plasma membrane (7). This internalization motif has been found to interact with several phosphotyrosine-binding domain (PTB) containing proteins such as Fe65 and X11, although phosphorylation within this motif is not required for binding (8–12). The interaction between the PTB domains of Fe65 or X11 proteins and the C terminus of APP has been shown to effect the distribution and turnover of APP, and the secretion of Aβ (13–16).

Mammalian X11 proteins (X11α, -β, and -γ) are homologues of *lin-10* in *Caenorhabditis elegans*. In conjunction with *lin-2* and *lin-7*, *lin-10* has been shown to be required for the precise targeting and localization of certain membrane proteins, such as the GLR-1 glutamate receptor (17, 18). Similar to *lin-10*, X11 proteins possess multiple protein interacting domains (see Fig. 1*A*) and have been ascribed to function as adaptor proteins that were critical for protein trafficking (19–23). It has therefore been postulated that X11 may modulate APP metabolism by altering the trafficking and distribution of APP (24). There were several lines of evidence to support a role of X11 proteins in the APP metabolic pathway. First, X11α and -β co-localize with APP in neurons and when expressed heterologously in cell lines (16, 24, 25). Second, mutations in the C terminus of APP that diminished interaction between X11α and APP also impaired the internalization of APP (12, 26). Third, overexpression of X11α or X11β proteins have been shown to increase the cellular APP level, increase release of a secreted form of APP (APPs), and inhibit Aβ secretion (13, 15, 16, 27–29). Although the mechanism is not known, several recent findings have suggested that these effects involved interactions of X11 with additional proteins. For instance, it has been shown that both X11α and -β can directly interact with presenilin-1, a protein that is genetically linked to γ-secretase activities, through their PDZ domains (30). Furthermore, the C-terminal PDZ domain of X11β was reported to suppress the NF-κB/p65-induced Aβ42 secretion and this effect did not require the binding of X11 to APP (28). Recently, a novel perinuclear/endoplasmic reticulum-localized protein termed XB51 was shown to interact with the N-terminal of X11β and abolished the interaction between APP and X11β (27).

Munc18a is a protein essential for Ca^{2+}-regulated exocytosis and membrane trafficking in neurons (31). Of importance, Munc18a also interacts with an MID domain at the N-terminal

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[1] The abbreviations used are: Aβ, β-amyloid peptide; APP, amyloid precursor protein; APPs, secreted N-terminal ectodomain of APP; APPs-β, secreted β-secretase cleavage product of APP; APPsw, double missense mutation of APP (K651N/M652L, positions based on APP_{751}) identified in a Swedish kindred of familial Alzheimer's disease; GST, glutathione *S*-transferase; HEK293 cells, human embryonic kidney 293 cells; His_6, epitope tag with a string of six histidine residues; MID, Munc18a interacting domain; PDZ, conserved binding motif initially found in post-synaptic density-95 (PSD-95), *Drosophila* Disks-large (Dlg), and epithelial tight junction protein zona occludens-a (ZO-1); PTB, phosphotyrosine-binding domain; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ANOVA, analysis of variance.

half of X11α and X11β, and therefore X11 proteins have also been named Mint (Munc18-interacting) proteins (32, 33). X11γ lacks an MID and exhibited no interaction with Munc18a in a yeast two-hybrid assay (33). The deletion of the N-terminal region including the MID from X11β has been shown to reduce its affinity to APP, even though the PTB domain for APP interaction remained intact (16). Deletion of a comparable region in X11α also abolished its effect in promoting APPs-β secretion (29).

X11α, X11β, as well as Munc18a show specific expression in the nervous system, however, the physiological consequence of their interaction within neurons was currently not known (34). The central focus of the current investigation was to test the hypothesis that Munc18a, through its interaction with X11, modulates APP metabolism and Aβ secretion. We have compared the actions of X11α, X11β, X11γ, and the N termini deleted mutants of X11α and -β on APP metabolism. We demonstrate that a strong synergistic effect exists between Munc18a and X11 proteins on APP processing. In addition, although the MID domain was sufficient and essential for a direct Munc18a interaction, we found that a portion of the synergistic effect was mediated via indirect interactions.

EXPERIMENTAL PROCEDURES

Expression Constructs—X11β, X11γ, Myc-tagged human X11α and its deletion mutants in pRK5 vector were obtained from Dr. Ben Margolis, University of Michigan. The Swedish mutant of APP (APPsw; K595N/M596L) was used in this study instead of wild-type APP to enhance the β-secretase activity in HEK293 cells to mimic neuronal APP metabolism and to increase the Aβ40 level in conditioned medium. It also facilitated analysis of β-secretase activity of transfected cells by the enzyme-linked immunosorbent assay described below. X11β, X11$\beta\Delta$N, X11γ, and rat Munc18a were subcloned into engineered pcDNA3.1 vectors with either a Myc tag or a His_6 tag at the N-terminal. Munc18a, His-X11β, His-X11γ, and His-X11βMID were subcloned into pGEX-KG vector to produce recombinant proteins in bacteria. The MID construct used in this study, which spans amino acid residues 179–282 of human X11β, covers the region aligned to the MID mapped in rat X11α (32).

Cell Culture and Transfection—HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Cells were replenished with fresh medium 2–4 h before transfection and they were transfected at ~30–50% confluence using a standard calcium phosphate precipitation protocol. Plasmid DNA constructs for transfection were prepared with a commercial kit (Qiagen). Plasmid vector pEGFP-C1 (CLONTECH) was used to monitor the transfection efficiency. Medium was changed 8–16 h after transfection.

β-Secretase Activity Measurements—Two days after transfection, the transfected cells were fed with fresh medium. After 2–4 h of conditioning, the medium was collected. β-Secretase activity was determined by quantitating the amount of β-secretase cleavage product of APPsw secreted into the medium using a APPs-β sandwich enzyme-linked immunosorbent assay as previously described (35). An affinity purified antibody (931) specific for the C terminus of the β-secretase cleavage product of APPsw was immobilized on the enzyme-linked immunosorbent assay plate and used to determine the APPs-β present in the media.

Protein Extraction and Western Blot Analysis—Cells were lysed 2 days after transfection in lysis buffer (50 mM HEPES, pH 7.5, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA) supplemented with protease inhibitors (1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were sonicated for 5 s with a high frequency cell disrupter and then centrifuged at 23,000 $\times$ *g* for 20 min at 4 °C. The supernatants were quantified for protein content using a commercial kit based on method of Bradford (41) (Bio-Rad). A fixed amount of protein was resolved by SDS-PAGE and transferred onto nitrocellulose membrane using a semi-dry protocol. Myc-tagged X11α, X11β, and their deletion mutants were detected using a monoclonal anti-Myc antibody (9E10) developed by J. M. Bishop, which was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences. Munc18a was detected with a monoclonal antibody m32320 (Transduction Laboratories). Both the endogenous APP and the heterologously expressed APPsw were detected with a polyclonal anti-APP antiserum C15TZ (a gift from Dr. Edward H. Koo, University of California, San Diego). Horseradish peroxidase-conjugated secondary antibodies and ECL reagent (Amersham Biosciences) were used to reveal the presence of proteins of interest. ECL signals were quantitatively detected with the Fluor-S Max imaging system (Bio-Rad). Student's *t* test and the single factor ANOVA test followed by Tukey multiple comparisons were used to analyze the difference among groups of data. Data were presented as mean ± S.E.

Immunoprecipitations—Cellular APP in freshly prepared cell lysates was immunoprecipitated using a mixture of 2 monoclonal antibodies (5A3 and 1G7) from Dr. Edward H. Koo. Myc-tagged X11α and X11$\alpha\Delta$N were immunoprecipitated using anti-Myc monoclonal antibody 9B11 from Cell Signaling Technology, Inc. After the cell lysates (1 mg in 500 μl) were incubated with the antibodies at 4 °C with gentle shaking for 2 h to overnight, antibody-bound protein complexes were pulled down using Protein G (for anti-APP) or Protein A (for anti-Myc) immobilized on agarose beads (Pierce). After washing three times in phosphate-buffered saline, the pellets were loaded and resolved on SDS-polyacrylamide gels together with their corresponding supernatants, final washes, and total cell lysates. The presence of APP, Munc18a, X11α, and X11$\alpha\Delta$N were detected by immunoblots with appropriate antibodies as described above.

Bacterial Protein Extraction and in Vitro Binding Assays—GST fusion protein constructs were transformed into *Escherichia coli* BL21RIL codon plus (Stratagene). Overexpression of fusion proteins was induced by 200 μM isopropyl-1-thio-β-D-galactopyranoside for 2–6 h at 37 °C and the bacterial proteins were extracted using a French Press approach. GST fusion proteins were isolated with glutathione-Sepharose beads (Amersham Biosciences). As initial experiments showed an interaction between the GST and Munc18a proteins, an in-frame His_6 tag was added between the GST tag and X11β coding sequences after the thrombin cleavage site in the construct. The GST tag was removed by thrombin treatment (6.25 units/250 ml of culture for 2–4 h at 20 °C) and the His_6 tag at the N terminus of the cleaved protein was used for affinity purification. Purified thrombin-cleaved His_6-labeled X11 protein (75 nM) was incubated with 1 nM to 5 μM purified Munc18a in binding buffer (25 mM Tris-HCl, pH 7.5, 40 mM NaCl, 0.5% CHAPS, 2.5 mM $MgCl_2$, 1 mM EGTA, 100 mg/ml phenylmethylsulfonyl fluoride) for 2 h at 4 °C. Munc18a bound to His_6-labeled X11 proteins was pulled down with TALON beads (CLONTECH). Pellets were washed three times by resuspending in binding buffer and centrifuged for 1 min at 800 $\times$ *g* at 4 °C. Supernatant from the final wash did not contain any Munc18a. The pellets were then resuspended in SDS sample buffer, and were resolved on an SDS-polyacrylamide gel. Munc18a was detected by immunoblotting quantitatively as described above. Data were fit to a single site binding curve using a software based algorithm (Prizm by GraphPad) and normalized to the calculated maximum binding values (B_{max}) from each experiment. Each binding curve was repeated 2–5 times.

RESULTS

The MID Domain Is Essential and Sufficient for Munc18a Interaction—The general domain structure of X11α, -β, and -γ and the deletion mutant constructs used in this study are illustrated in Fig. 1*A*. The PTB and PDZ domains were highly conserved among the three members of the X11 family with sequence identities between 60 and 90%, although their N-terminal regions differ drastically. The region of X11α essential for Munc18a interaction has been mapped to the MID region in the N-terminal half of the protein (residues 226–314) by yeast two-hybrid assay using overlapping rat cDNA clones (32). This region was aligned to amino acid residues 196–266 in human X11β (Fig. 1, *A* and *B*). Because of the diverse sequences at the first half of this region, the N terminus of MID in X11β was not well defined. Within the MID region, there was a stretch of amino acid sequence, (D/E)QEEDIDQIVAE, highly conserved between X11α and X11β in human, mouse, and rat (Fig. 1*B*). A BLAST search using the peptide sequence from this area did not pull out other known protein sequences. Such a consensus motif was absent in X11γ, whereas in X11β, there was an additional partial repeat of motif DQEED in the upstream sequence.

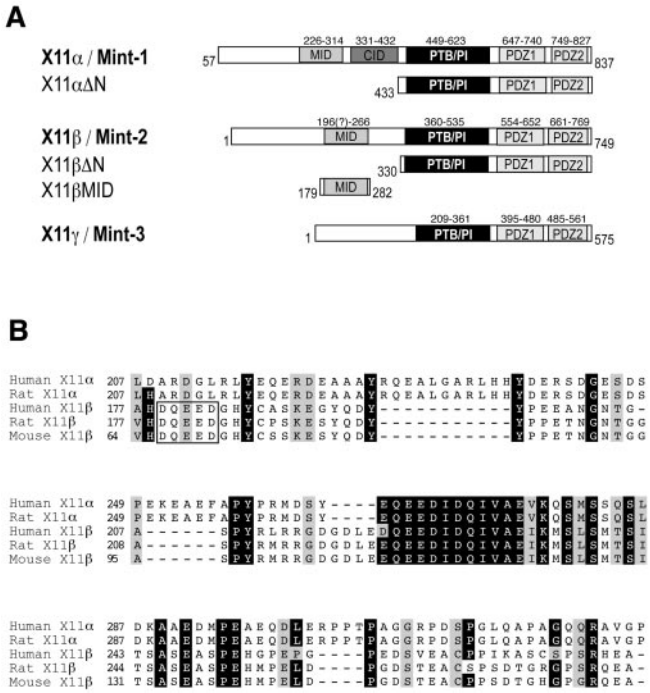

FIG. 1. **Domain structure of human X11 proteins and deletion mutants.** *A*, the MID was present at the N-terminal half of X11α (amino acid residues 226–314) and X11β (residues ~196–266). The N-terminal boundary of X11β was not well defined. X11γ does not have a MID but, like X11α and -β, it carries one PTB domain and two PDZ domains. The locations of these domains in human amino acid sequences based on alignment were labeled on *top* of each linear map. Within these interacting domains, X11α and X11β share 80–90% identical sequence. Sequence comparison to X11γ shows greater levels of heterogeneity (60–71% identical to X11α and -β). The mammalian expression constructs used in this study were either Myc-tagged (X11α, X11β, X11$\alpha\Delta$N, X11$\beta\Delta$N, and MID) or His-tagged (X11γ) at the N termini. The bacterial expression constructs for X11β and MID contain a His tag. The "full-length" Myc-X11α used in this study does not contain the first 56 amino acid residues, however, it has been shown to be functional in modulating APP processing (13, 29). *B,* protein alignments showed that a region homologous to the MID of X11α was present in X11β with an overall 32.4% sequence identity. The N-terminal boundary of MID in X11β was not well defined. The highly conserved motif ((E/D)QEEDIDQIVAE) was found in the middle of this region. Amino acid residues common for X11α and X11β are highlighted in *black*. Positions with conservative substitutions are marked with *gray shadow*. In addition, an additional partial repeat (DQEED) of the conserved motif present in all known mammalian X11β sequences was enclosed in a *box*.

Immunoprecipitation of Munc18a with Myc-tagged X11α overexpressed in HEK293 cells indicates that these two proteins can interact *in situ* (Fig. 2*A*). Such interaction required the presence of the MID-containing N terminus of X11α, suggesting that the MID domain was essential for X11-Munc18a interaction. This finding was consistent with a previous report using a yeast two-hybrid assay (33).

To establish the binding relationship between X11β and Munc18a, each protein was expressed as an epitope-tagged fusion protein in bacteria and purified for analysis of *in vitro* binding. Fig. 2*B* shows the binding relationship between Munc18a and X11β ($n = 5$). The EC_{50} was determined to be 281.5 nM. A similar binding assay determined a low level of interaction between His-tagged X11γ and Munc18a ($EC_{50} = 3.5$ μM), however, control experiments performed without X11 protein also exhibited a low level of nonspecific binding of Munc18a to the TALON beads ($EC_{50} = 7.5$ μM). This finding was consistent with a previous report that X11γ (Mint3) and Munc18a do not interact in a yeast two-hybrid assay (33). The low affinity of the MID-deficient X11γ to Munc18a was consistent with the idea that the MID of X11 proteins was essential for high affinity Munc18a binding. To further establish if the MID alone was sufficient for Munc18a interaction, a stretch of cDNA encoding 104 amino acids (residues 179–282 of X11β; see Fig. 1, *A* and *B*) was amplified from X11β by PCR and subcloned into an engineered pGEX-KG vector with an in-frame His_6 tag sequence. The EC_{50} of Munc18a interaction with this MID was determined *in vitro* to be 268.3 nM (Fig. 2*B*). The binding curves of X11β and MID to Munc18a were, therefore, essentially superimposable. These data together with the very low specific binding of Munc18a to X11γ suggest that the MID of X11 proteins was both essential and sufficient for the full binding capacity of Munc18a.

X11α Co-immunoprecipitated with APP—It has been shown that the X11α protein can interact with the C terminus of APP through its PTB domain (8). It has also been suggested that an N-terminal binding partner of X11β may modulate the affinity between APP and X11β (16). Because Munc18a specifically interacts with the MID domain at the N termini of X11α and X11β, we examined the effect of Munc18a on the APP-X11 interaction. APP was transfected alone or together with Myc-tagged X11α in the presence or absence of Munc18a into HEK 293 cells. APP was then immunoprecipitated using monoclonal antibodies specific for the mid-region of APP that was within the extracellular portion of APP and away from the intracellular X11α interacting domain at the C terminus. As shown in Fig. 2*C*, APP immunoprecipitation led to co-immunoprecipitation of Myc-tagged X11α protein. Although X11α was co-precipitated, a significant amount of X11α remained within the supernatant fraction. With the addition of Munc18a transfection, the amount of co-precipitated X11α was increased 8.4-fold (Fig. 2*C*). Surprisingly, the APP content in the lysates of triple transfected APPsw-Munc18a-X11α cells was also substantially higher compared with those cells co-transfected with APPsw and X11α leading to a 6.9-fold increase in immunoprecipitated APP. The increase in the co-precipitated X11α with co-expression of Munc18a was, therefore, proportional to the amount of APP present in the precipitate.

Effect of X11α on Cellular APP Levels Modulated by Munc18a—It has previously been shown that X11α stabilizes APP and prolongs its half-life (13). Our data above suggested that Munc18a may act to further promote stabilization of APP. To determine whether co-expression of Munc18a with X11α potentiated cellular APP content, we quantitatively compared APP levels in the lysates of transfected cells. Our initial results confirmed that co-expression of X11α with APPsw in HEK293 cells increased the cellular APP levels (3.84 ± 0.92-fold of APPsw control, $n = 7$; Fig. 3, *panels A* and *B*). The increased level of cellular APP was observed in both mature and immature form, but was more prominent in the latter (2.39 ± 0.43-fold for mature form *versus* 4.31 ± 1.42-fold for immature form; $n = 7$). In the absence of X11α, Munc18a by itself showed no effect on the cellular APP level (1.03 ± 0.10-fold of control, $n = 7$; Fig. 3, *panels A* and *B*). In contrast, co-expression of Munc18a and X11α in APPsw expressing HEK cells boosted the cellular APP level to 13.94 ± 0.88-fold of that observed in control cells transfected with APPsw alone. In other words, there was a 3.6-fold further increase (or a net increase of 10.1 times of control level) in cellular APP as a result of Munc18a co-expression. Such an additional increase in the APP level did not correlate to the small and insignificant increase in the X11α level caused by Munc18a expression (1.00 ± 0.07 *versus* 1.24 ± 0.07; $n = 8$; $p > 0.1$, paired *t* test; Fig. 3*C*). In addition to the effect of X11α on cellular APP level, we also observed that the protein level of overexpressed Munc18a was increased

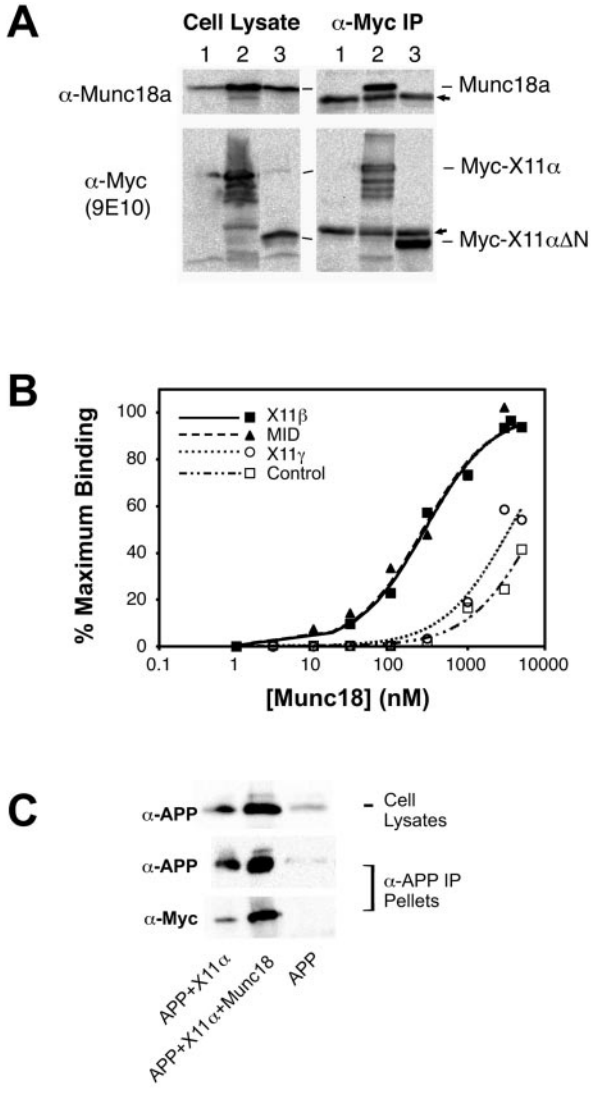

FIG. 2. **X11 interaction with Munc18a and APP.** *A,* Munc18a co-immunoprecipitated with X11α but not X11$\alpha\Delta$N. Munc18a was transfected alone (*lane 1*), with Myc-tagged X11α (*lane 2*), or with Myc-tagged X11$\alpha\Delta$N (*lane 3*) into HEK 293 cells. Expressions of corresponding proteins were visualized by immunoblots of the cell lysates shown on the *left panels*. Myc-tagged X11α and X11$\alpha\Delta$N were immunoprecipitated from cell lysate with an anti-Myc (α-Myc) monoclonal antibody 9B11 (*right panels*). *Upper panels* show immunoblots using an anti-Munc18a (α-Munc18a) monoclonal antibody. *Lower panels* show immunoblots with α-Myc monoclonal antibody 9E10. The bands at the *arrows* on *right panels* are the heavy chain of mouse IgG from 9B11 and indicate that an equal amount of antibody was present in all immunoprecipitated pellets. Co-immunoprecipitation of Munc18a with X11α requires the MID-containing N terminus of X11α. *B*, *in vitro* binding reactions comparing the binding between Munc18a and X11β (■, $n = 5$), MID of X11β (▲, $n = 2$), and X11γ (○, $n = 3$). There was no observable difference between the X11β and the MID binding curves, whereas the binding curve for X11γ was similar to the TALON bead control (□, $n = 2$). *C,* effects of Munc18a on the APP level and on co-immunoprecipitation of X11α with APP. APPsw, Myc-tagged X11α, and Munc18a were expressed in HEK293 cells and APP was immunoprecipitated from the cell lysates with a monoclonal antibody targeted to the mid-region of APP. *Top panel* shows an immunoblot of cell lysates using anti-APP antiserum (α-APP) to show the relative amount of APP present in the cells transfected with different combinations of expression constructs. The *bottom two panels* show Western blots of anti-APP immunoprecipitated proteins probed with α-APP and α-Myc antibodies. The cellular APP level present in the cell lysates was elevated by the co-expression of X11α and further increased by the addition of Munc18a. As a result, the amount of precipitated APP increased accordingly. The levels of co-precipitated Myc-X11α were also increased by the addition of Munc18a proportional to the amount of precipitated APP. There was no discernible APP or X11α present in aliquots of the final wash. The experiment was repeated twice with identical observations.

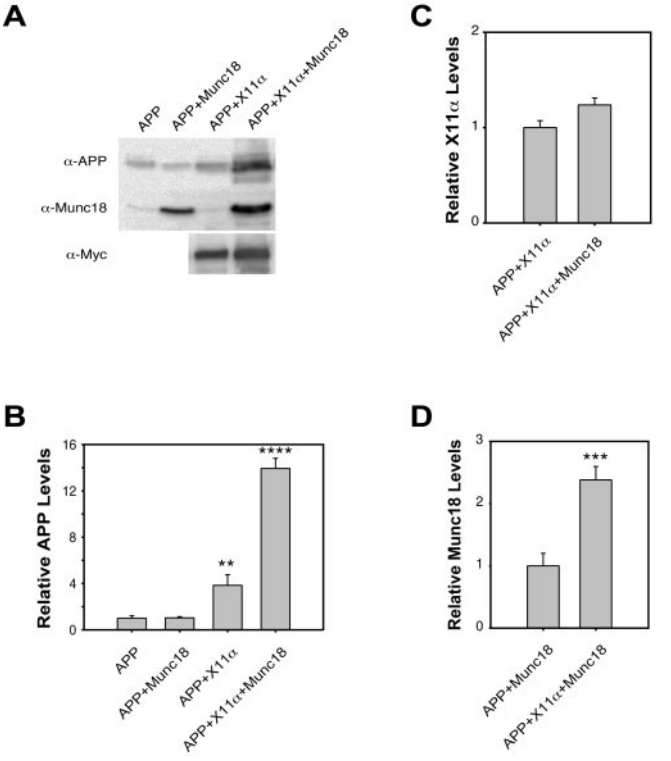

FIG. 3. **Regulation of APP metabolism by X11α and Munc18 in HEK293 cells.** HEK293 cells expressing different combinations of APPsw, His-Munc18, and Myc-X11α were analyzed for APP, X11α, and Munc18 protein levels. *A*, immunoblots comparing the cellular APP level in the presence of X11α, Munc18a, or X11α + Munc18a. Munc18a alone had little effect on the APP level of the cell, however, it exerted a synergistic effect with X11α in enrichment of cellular APP. The amplitude of the synergistic effect cannot be accounted for by the insignificant increase in the Myc-X11α expression. *B*, quantitative comparison of relative cellular APP content under the various treatments. ECL signals were quantified using the Fluor-S Max (Bio-Rad) imager. Data from seven replicated experiments were normalized to the mean cellular APP level of the control group (APPsw transfected alone). One factor ANOVA followed by Tukey multiple comparisons showed a highly significant difference in APP levels among different treatments. Munc18a co-expression showed no effect on the cellular APP level, whereas the expression of X11α elevated the APP level significantly (**, $p < 0.025$). The combination of X11α and Munc18a increased the APP level to 13.94 ± 0.88-fold of control (****, $p < 0.001$, $n = 7$). *C*, comparison of expression levels of X11α in the cell lysates with and without Munc18a co-expression. The addition of Munc18a did not significantly alter the level of X11α. *D*, effect of X11α co-expression on Munc18a levels in cell lysates. The presence of X11α resulted in a significant increase in the level of Munc18a in the transfected cell ($n = 6$; $p < 0.005$, paired *t* test).

by 1.4-fold when they were co-transfected in the HEK cells ($p < 0.005$; Fig. 3*D*).

Effect of X11β and X11γ Proteins on Cellular APP Levels—Similar to X11α, the X11β and X11γ isoforms also contain a PTB domain and have been shown to interact with APP by yeast two-hybrid and co-immunoprecipitation assays (16, 33, 36). It has also been demonstrated that X11β may inhibit the secretion of Aβ40 in APP695 stably transfected HEK293 cells (16). The effects of X11β on the cellular APP levels and the action of X11γ on APP processing have not, however, yet been established. Fig. 4*A* shows that X11β increased the cellular level of APP by 61.3% ($p < 0.05$) when it was co-transfected with APPsw into HEK293 cells. In contrast to X11α and β, X11γ had no observable effect on cellular APP content (117 ± 24% of control, $n = 7$), even though it contains the PTB domain and interacts with the C terminus of APP. Because the major structural difference between X11α/β and X11γ was at the N-terminal half of the protein where the MID resides, these data suggest that Munc18a interacting at the N-terminal of

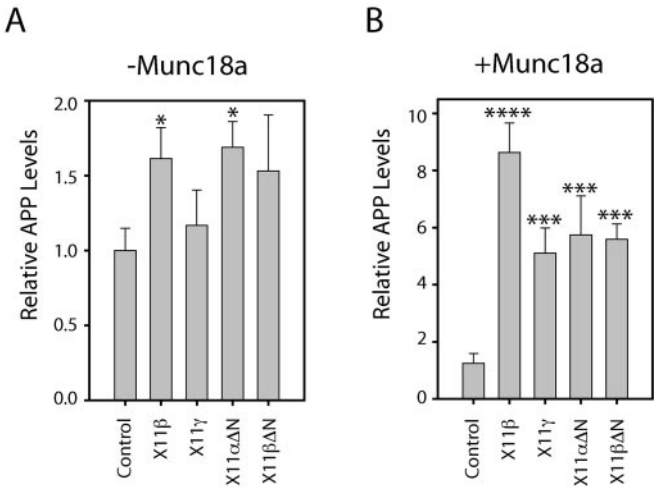

FIG. 4. **Comparison of the effects of X11β, X11γ, and N termini deleted X11 mutant proteins on relative cellular APP content in the absence (*A*) and presence (*B*) of Munc18a co-expression.** In the absence of Munc18a (*A*), X11β showed a small increase in cellular APP level when co-transfected with APPsw into HEK cells (*, $p < 0.05$), whereas X11γ did not show an effect on APP level. The X11$\alpha\Delta$N also displayed a partial effect of X11α with a small but significant elevation of cellular APP level (*, $p < 0.05$, paired *t* test), whereas the increase in cellular APP level by the expression of X11$\beta\Delta$N was not significant. In the presence of Munc18a (*B*), however, both X11β and X11γ displayed highly significant effects on the cellular APP levels (****, $p < 0.001$, and ***, $p < 0.005$, respectively). With Munc18a, X11β has a much stronger effect on APP retention than X11γ ($p < 0.01$, Tukey test). In addition, both X11$\alpha\Delta$N and X11$\beta\Delta$N also significantly increased the cellular APP levels over control (***, $p < 0.005$, Tukey test). As a result, the N terminus deletion mutants of X11α and X11β become indistinguishable from X11γ in their actions on cellular APP retention. All data were normalized to the average APP level of the control cells transfected with APPsw alone.

X11α/β may be crucial for the effects of X11 on APP processing and support the idea that direct interactions between Munc18a and X11α/β may modulate the activity of X11 on APP processing.

Effect of Munc18a with X11β and -γ—Co-expression of Munc18a with X11α resulted in a potentiating effect on cellular APP levels. Therefore, we next examined if such an effect would be observed with the co-expression of X11β (which possesses an MID) or X11γ (which lacks an MID) with Munc18a. Fig. 4*B* shows that when Munc18a was expressed together with X11β, the APP content of the cell became 8.6-fold of the APPsw control. Co-expression of Munc18a then led to a 5.4-fold greater increase of the already elevated APP level in X11β expressing cells (a net increase of 8 times control level). The extent of this Munc18a potentiating effect was comparable with that observed with X11α. Interestingly, co-expression of Munc18a with X11γ also increased the APP level by 4.1-fold, although X11γ by itself showed no significant effect on cellular APP level. The net difference in APP levels of X11γ expressing cells by the addition of Munc18a expression was 3.9-fold of the control level (compared with 10.1-fold with X11α and 8-fold with X11β). The X11γ results suggested that in addition to direct functional effects of Munc18a binding on APP levels, there may also be indirect functional interactions between Munc18a and X11 proteins.

Effects of N-terminal Deleted Mutants of X11α and -β on Cellular APP Level—As shown in Fig. 1*A*, the N-terminal halves of the three members of the X11 family exhibit considerable heterogeneity. To examine whether the differential effects among X11α, -β, and -γ were because of their N-terminal sequence and to verify whether the MID-containing N-terminal motifs of X11α and X11β were required for the synergistic effect of Munc18a, N-terminal deletion mutants of X11α and X11β (namely X11$\alpha\Delta$N and X11$\beta\Delta$N) were tested for their effects on APP metabolism. For the X11$\alpha\Delta$N, the first 432 amino acids encompassing the MID and the CASK interacting domain were removed, whereas the PTB and PDZ domains remained intact. Similarly, the N-terminal 329 amino acids were removed from X11β in the X11$\beta\Delta$N construct and left the highly conserved PTB and PDZ domains. The X11$\alpha\Delta$N and X11$\beta\Delta$N proteins shared 83% identical sequence, whereas they were 59 and 55% identical to X11γ, respectively.

As shown in Fig. 4*A*, when co-transfected with APPsw into HEK cells, X11$\alpha\Delta$N increased the cellular APP level by only 69 ± 17% ($n = 5$; compared with 284% with the wild-type X11α). This finding suggested that the N-terminal of X11α carries additional signaling motifs that participate in modulating APP metabolism. The N-terminal deletion had little effect on the action of X11β on cellular APP levels (53 verses 61% with full-length X11β), but it decreased its responsiveness to the potentiating effect of Munc18a. As the X11$\alpha\Delta$N and X11$\beta\Delta$N share 83% identical sequence, it was not surprising that they showed an almost identical effect on cellular APP level. Of particular interest, Munc18a was found to increase the cellular APP content by an additional 3.4- and 3.7-fold when it was coexpressed in HEK cells with X11$\alpha\Delta$N or X11$\beta\Delta$N, respectively (compared with 3.6 and 5.4-fold with X11α and X11β). The N-terminal deletions of X11α and X11β made them indistinguishable from X11γ in their effects on APP metabolism when the mutants were co-expressed with Munc18a. This finding suggests that the structural differences at the N-terminal halves of the X11 proteins may explain their different efficacies in retaining cellular APP and confirms that an additional indirect functional interaction occurs between Munc18a and X11 proteins that does not involve the MID region.

Effect of X11α and Munc18a on Aβ-secretion—X11α and X11β have been shown to suppress secretion of Aβ peptides from APP-transfected HEK cells (13, 16). Earlier in this report, we showed that Munc18a potentiated the actions of X11 proteins in retaining cellular APP. Such synergistic action was also exhibited in the suppressive action of X11α on Aβ40 secretion (Fig. 5). The co-expression of X11α reduced the Aβ40 secretion of the APPsw expressing cell by 70.0 ± 6.1% ($n = 7$, $p < 0.001$), whereas co-expression of Munc18a did not exert an effect. Surprisingly, the co-expression of both X11α and Munc18a with APPsw dramatically decreased the Aβ40 secretion level by 97.3 ± 2.1% ($n = 3$, $p < 0.001$). In other words, the constitutive secretion of Aβ40 was almost completely abolished. To investigate whether the direct interaction between X11α and Munc18a was involved, the experiment was repeated with X11$\alpha\Delta$N in place of X11α. As previously reported (29), X11$\alpha\Delta$N was capable of suppressing Aβ40 secretion to 23.2 ± 9.8% of control ($n = 3$, $p < 0.01$). The coexpression of Munc18a and X11$\alpha\Delta$N further decreased the Aβ40 secretion to 3.9 ± 0.8% ($n = 3$, $p < 0.001$) of the control APPsw expressing cell. The similar effect of Munc18a coexpression with X11α or X11$\alpha\Delta$N indicated that the direct X11-Munc18a interaction was not required for the potentiating effect of Munc18a in suppressing Aβ40 secretion.

Effect of X11 Proteins and Munc18a on β-Secretase Activity—The decrease in Aβ secretion by X11 and Munc18a suggested a reduction in APP processing in either the β-secretase or γ-secretase pathway, for instance, by redistributing the APP within the cell. A previous report showed that X11α increased APPs, presumably mainly APPs-β, secreted into medium and that such an increase required the N-terminal of X11α (29). This finding provided indirect evidence that the reduction in

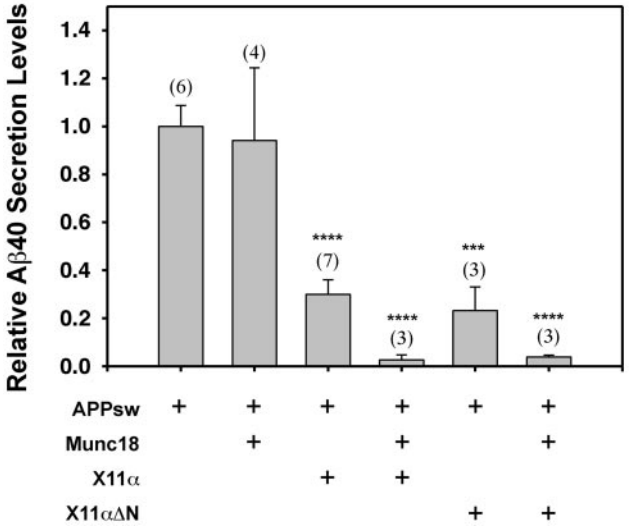

FIG. 5. **Suppression of Aβ40 secretion by X11α and Munc18a.** X11α significantly decreased the Aβ40 secretion from APPsw expressing HEK293 cells (****, $p < 0.001$). X11αΔN exhibited a similar effect indicating that such activity did not require the presence of the N-terminal sequence (***, $p < 0.005$). Expression of Munc18a in the absence of X11 proteins did not show an effect on Aβ40 secretion, however, the coexpression of Munc18a and X11α almost completely abolished Aβ40 secretion from APPsw expressing cells ($p < 0.001$). This enhancement of the suppressive action of X11α did not involve the direct physical interaction between X11α and Munc18a because the action of X11αΔN was also boosted by the coexpression of Munc18a. Data were analyzed with single factor ANOVA followed by Tukey test compared with the control group.

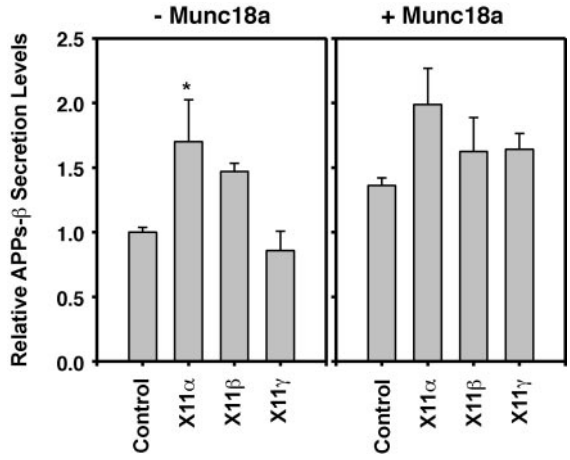

FIG. 6. **Effects of X11 proteins and Munc18a on APPs-β secretion.** The secretion levels of APPs-β, which was derived from the heterologously expressed APPsw, under the co-expression of various X11 proteins were examined. *Left panel*, in the absence of Munc18a, X11α showed a significant increase in APPs-β secretion (*, $p < 0.05$, $n = 4$). A smaller increase by X11β was also observed ($p < 0.05$, paired t test, $n = 3$), whereas X11γ showed no effect on APPs-β secretion. *Right panel*, Munc18a alone also showed an elevation in the APPs-β secretion from cells expressing APPsw ($p < 0.05$, paired t test, $n = 3$), however, it did not potentiate the effects of X11α and X11β in APPs-β secretion. Data were analyzed by one factor ANOVA followed by Tukey test unless otherwise specified.

Aβ secretion was a result of reduced γ-secretase activity, however, the potentiating effect of Munc18a may still be mediated by the suppression of either secretase. Here we examined the effect of the three X11 proteins and Munc18a on β-secretase activity by comparing the level of APPs-β derived from the heterologously expressed APPsw secreted into the conditioned medium using an enzyme-linked immunosorbent assay (Fig. 6). In the absence of Munc18a, X11α increased the level of APPs-β secreted into conditioned medium (169.6 ± 31.5%, $n = 4$, $p < 0.05$). Smaller increases in APPs-β secretion by X11β (145.6 ± 6.4%, $n = 3$) and Munc18a (136.3 ± 7.8% ($n = 4$), but not X11γ (84.9 ± 14.3%, $n = 3$), were also revealed by the two-tailed paired t test (*i.e.* $p < 0.05$). Munc18a showed no significant further enhancement in APPs-β secretion in cells expressing X11α and X11β. The coexpression of Munc18a with X11γ increased the APPs-β secretion to 162.8 ± 13.2% of control which was not significantly stronger than the effect of Munc18a alone. Because Munc18a did not exhibit an inhibitory effect, but rather a stimulatory effect, on β-secretase activity, the further suppression of Aβ40 secretion by Munc18a coexpression can only be explained by a reduction in γ-secretase processing of APP and its metabolites.

DISCUSSION

Previous studies have shown that X11α and X11β proteins increase the cellular APP level, increase APPs release, and inhibit Aβ secretion (13, 15, 16, 27–29). A protein factor interacting at the N terminus of X11β had been postulated to modulate the interaction between X11β and APP and to provide regulation of these events (16, 28). The present investigation demonstrates that Munc18a, which interacts with X11α and X11β at their N termini, strongly potentiates the effect of X11 proteins in suppressing APP catabolism. In addition, we have found that Munc18a functionally regulates X11 actions on APP metabolism through a combination of direct and indirect interactions with the X11 proteins. The direct effect of Munc18a on X11 regulation of APP processing occurs through interaction with the MID located at the N terminus of X11α and X11β. The indirect effects of Munc18a required co-expression of the X11 proteins but occurred independent of the MID, as demonstrated by the co-expression of Munc18a with X11αΔN and X11βΔN constructs. Furthermore, we have identified and characterized a novel action of X11γ in APP processing that involves the indirect interaction with Munc18a. Last, the indirect functional interaction between Munc18a and X11α was capable of almost completely abolishing the constitutive release of Aβ40 by suppressing a γ-secretase pathway.

Actions of X11γ on APP Metabolism—Similar to X11α and X11β, X11γ also contains a PTB domain that shares over 60% identical sequence to those in X11α and X11β and has been shown to interact with APP (8, 10, 36). The physiological significance of interactions between APP and X11γ in APP metabolism has, however, not been reported. In the present study, we show that co-expression of X11γ with Munc18a increased the cellular level of APP and APPs-β secretion, however, X11γ exerted no effect in the absence of Munc18a. This finding indicates that the PTB domain of X11 proteins was not sufficient for modulating APP metabolism and suggests an indirect functional interaction between X11γ and Munc18a. Because X11γ differs considerably from the other two X11 proteins at the N terminus, such structural differences may explain the lack of activity by X11γ.

Synergistic Effect of Munc18a and X11 Proteins on APP Processing—We demonstrate that co-expression of Munc18a potentiated the action of X11 proteins in elevating cellular APP levels, whereas Munc18a overexpression alone in APPsw expressing cells did not augment the cellular APP content. There were three possible mechanisms to account for such a synergistic effect. First, as X11 protein expression alone was capable of increasing cellular APP levels, Munc18a may enhance the action of X11 proteins by increasing the cytosolic X11 protein levels. However, comparison of the expression levels of X11 proteins in the presence or absence of Munc18a showed that there were not significant changes in the X11 protein levels. Second, interaction of Munc18a with X11 at MID may trigger

an allosteric effect on the PTB domains of X11 such that the affinity of X11α and X11β for APP was greatly increased. The increased affinity may enhance the actions of X11 in suppressing APP processing and $\alpha\beta$ secretion. Indeed, Tomita *et al.* (16) have suggested that an N-terminal domain of X11 may provide this type of allosteric and functional regulation. Consistent with this model, we demonstrated that co-expression of Munc18a with X11α led to greatly enhanced co-immunoprecipitation of X11α with APP. Yet, we also observed an increase in the total cellular APP content by Munc18a in the presence of X11α. Therefore, the increase in APP content alone under these experimental conditions was likely sufficient to fully account for the observed increase in X11α co-precipitated with APP. Additional investigations will, however, be required to definitively establish that the effects do not result from a change in X11 affinity for APP upon interaction with Munc18a. A third possible mechanism consistent with our findings was that there are direct physical and/or indirect functional interactions between Munc18a and X11 proteins. In this model the two proteins may either work simultaneously or sequentially in a common pathway.

Direct and Indirect Functional Interactions between Munc18a and X11 Proteins—Using immunoprecipitation and *in vitro* protein binding approaches we show that the MID region of X11 was essential and sufficient to constitute a direct interaction between Munc18a and X11 proteins. This direct physical interaction provides a molecular basis for the functional interaction between the two proteins in modulating APP processing. It has previously been shown that an N-terminal deletion of X11β that removed the MID region suppressed the interaction between X11β and APP (16). Binding of XB51 to the N terminus of X11β has also been shown to suppress the binding of X11β to APP through an allosteric interaction (27). In this study we demonstrate that the N termini of X11α and X11β was required for the full effect of Munc18a on APP processing. These findings strongly support a role for direct Munc18a-X11 interaction in regulating APP metabolism. Such direct interaction facilitates the retention of cellular APP but was not involved in modulating the Aβ40 secretion pathway.

We provide evidence that indirect interactions also occur between Munc18a and X11 proteins that exhibit strong actions on APP metabolism. In this regard, Munc18a co-expression with the X11$\alpha\Delta$N and X11$\beta\Delta$N mutants or X11γ lacking the MID domain was still capable of inhibiting APP metabolism, albeit at a level slightly lower than with the full-length X11 protein. This indirect functional interaction was also observed in the inhibition of Aβ40 secretion and likely involved additional interactions through other proteins. Interestingly, X11γ was recently shown to interact with APP indirectly through other proteins besides the direct interaction at its PTB domain (37). These additional interaction mechanisms may ensure the co-segregation of different proteins within the appropriate subcellular domains and trafficking pathways. In addition, the indirect functional interaction(s) may be mediated by sequential actions of Munc18a and X11 proteins in a common metabolic pathway. As Munc18a overexpression alone failed to affect cellular APP levels or Aβ40 secretion, the sequential action model would require effects of X11 to be exerted prior to Munc18a.

Model for the Actions of Munc18a and X11α on APP Metabolism—X11 proteins exert multiple actions on APP metabolism: retaining cellular APP, stimulating β-secretase activity, and suppressing Aβ secretion (13, 16). Different parts of the X11 protein may be involved in different actions. For instance, the N terminus of X11α has been reported to be critical in promoting APPs secretion, but not essential for suppressing Aβ release (29). The C-terminal PDZ domains of X11α and X11β can interact with presenilin-1 and such interactions may be related to its suppressive effect in γ-secretase pathway. Because the APP processing pathways were highly compartmentalized within the cell, the various actions of X11α proteins may take place at different locations (3, 5, 6, 13, 16, 24, 28–30, 38). Likewise, Munc18a may have differential effects in different steps of APP cellular trafficking pathways, and some may involve the direct interaction at the N termini of X11 proteins. Fig. 7 illustrates proposed roles of Munc18a in APP processing.

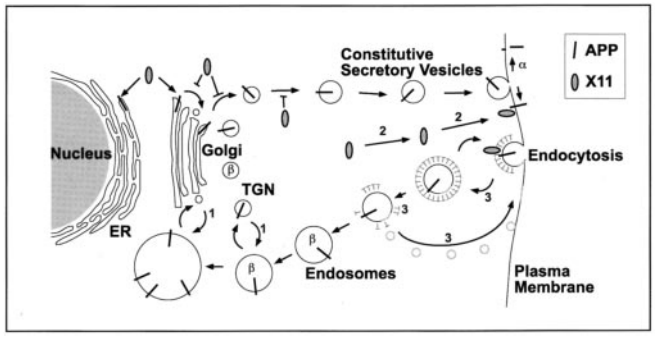

FIG. 7. **Model showing proposed sites of Munc18a actions in APP processing.** APP synthesis in endoplasmic reticulum (*ER*) was followed by post-translational processing primarily as it was being transported through the Golgi apparatus. APP was then directed to the constitutive secretion pathway where it reaches the cell surface. A certain amount of APP was directed to the endosome-lysosome pathway from the Golgi. APP that reaches the cell surface will either be processed by plasma membrane delimited α-secretase or re-internalized into endosomes where β-cleavage may occur. X11 may increase the cellular APP level by blocking the maturation of APP and trapping it in the perinuclear region. On the other hand, X11-APP interaction may facilitate the internalization of APP and decrease its exposure time on the cell surface. Three actions are proposed for Munc18a regulation. 1) It may help shuttle the APP trapped in Golgi or trans-Golgi network (*TGN*) to other vesicle pools and increase the capacity for retaining cellular APP. 2) The direct interaction between X11 and Munc18a may help to bring more X11 to the cell membrane and facilitate the re-internalization of APP. 3) Munc18a may be involved in the recycling of other vesicles that, at the same time, may facilitate the endocytic pathway of APP.

One action of X11α may be mediated by blocking the maturation and trafficking of APP in the early pathway such as in the Golgi apparatus and the trans-Golgi network as indicated by the co-localization of overexpressed APP and X11α in the perinuclear region in Chinese hamster ovary cells (24). This was consistent with the observation that X11α expression led to a more prominent increase in the immature than the mature form of APP. The APP trapped in the perinuclear region may be less susceptible to enzyme degradation leading to an increase in cellular APP content. Munc18a has been shown to interact with SNAP (soluble NSF attachment protein) receptor proteins which regulate trafficking of synaptic vesicles to the release sites as well as other vesicles between different intracellular compartments (31, 39). The presence of Munc18a may help to transport the trapped APP away from the Golgi or trans-Golgi network to other vesicle pools, and therefore, increase the capacity for APP storage within the cell as well as shuttling to a compartment that carries β-secretase activity. Such redistribution of APP may prevent the APP from entering the γ-secretase processing pathway leading to the decrease in Aβ secretion.

On the other hand, the internalization of APP has been linked to the X11α-APP interaction (12, 26), although the biological consequence of such an interaction at the plasma membrane remains unclear. Through interaction with Munc18a, the distribution of X11 proteins may be altered. For example, more X11 protein may be directed to the plasma membrane and lead

to enhanced action of X11 in the internalization of APP. Munc18a may also participate in the mechanism to transport the reinternalized APP to various intracellular compartments. Last, APP has been shown to co-internalize with synaptic integral membrane proteins (40). The presence of Munc18a may facilitate the recycling of the synaptic vesicle proteins and the internalization of APP as well. It will be critical to elucidate the pathway(s) through which Munc18a exerts direct or indirect regulation of the action of X11 in APP processing.

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