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Inhibition of Glycosphingolipid Biosynthesis Reduces Secretion of the β-Amyloid Precursor Protein and Amyloid β-Peptide*[S]

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Alzheimer disease is associated with extracellular deposits of amyloid β-peptides in the brain. Amyloid β-peptides are generated by proteolytic processing of the β-amyloid precursor protein by β- and γ-secretases. The cleavage by secretases occurs predominantly in post-Golgi secretory and endocytic compartments and is influenced by cholesterol, indicating a role of the membrane lipid composition in proteolytic processing of the β-amyloid precursor protein. To analyze the role of glycosphingolipids in these processes we inhibited glycosyl ceramide synthase, which catalyzes the first step in glycosphingolipid biosynthesis. The depletion of glycosphingolipids markedly reduced the secretion of endogenous β-amyloid precursor protein in different cell types, including human neuroblastoma SH-SY5Y cells. Importantly, secretion of amyloid β-peptides was also strongly decreased by inhibition of glycosphingolipid biosynthesis. Conversely, the addition of exogenous brain gangliosides to cultured cells reversed these effects. Biochemical and cell biological experiments demonstrate that the pharmacological reduction of cellular glycosphingolipid levels inhibited maturation and cell surface transport of the β-amyloid precursor protein. In the glycosphingolipid-deficient cell line GM95, cellular levels and maturation of β-amyloid precursor protein were also significantly reduced as compared with normal B16 cells. Together, these data demonstrate that glycosphingolipids are implicated in the regulation of the subcellular transport of the β-amyloid precursor protein in the secretory pathway and its proteolytic processing. Thus, enzymes involved in glycosphingolipid metabolism might represent targets to inhibit the production of amyloid β-peptides.

The deposition of amyloid β-peptides (Aβs)[1] in extracellular plaques is an invariant neuropathological feature of Alzheimer disease (AD) (1, 2). Aβ derives from the β-amyloid precursor protein (APP) by proteolytic processing, which involves sequential cleavages by proteases called β- and γ-secretases (1, 3, 4). APP is a type I membrane protein that is transported from the endoplasmic reticulum via the Golgi compartment to the cell surface and undergoes maturation by *N*′- and O′-glycosylation (1, 3, 4). Within the secretory pathway and at the cell surface APP is predominantly cleaved by α-secretase, resulting in the secretion of soluble APP (APP_S) (5). Because α-secretase cleaves APP within the Aβ domain, this cleavage precludes the generation of Aβ. Alternatively, APP can be cleaved by β-secretase. The cleavage of APP by β-secretase occurs predominantly in endosomal and lysosomal compartments after internalization from the cell surface (6, 7). The C-terminal fragments (CTFs) of APP resulting from α- or β-secretase cleavage can be cleaved within the transmembrane domain by γ-secretase to release p3 and Aβ, respectively (1, 3, 8).

The proteolytic processing of APP is influenced by the lipid composition of cellular membranes, as demonstrated by the pharmacological modulation of cellular cholesterol levels (9–12). In addition, the inhibition of acyl-coenzyme A:cholesterol acyltransferase (ACAT) also led to strong reduction of Aβ generation in cultured cells and transgenic mice, indicating that cholesterol esters also influence proteolytic processing of APP (13, 14). These effects on APP processing might involve altered cleavage of APP by α-secretase and/or β-secretase, probably by redistribution of APP and secretases between distinct membrane microdomains (15–18).

Apart from cholesterol, glycosphingolipids (GSLs) have also been implicated in the pathogenesis of AD. It has been shown that the levels of several gangliosides are altered in AD brains (19). In addition, the ganglioside GM1 binds to Aβ and might contribute to early deposition of the peptide in amyloid plaques (20–22).

The biosynthesis of GSLs starts with the generation of glucosylceramide from UDP-glucose and ceramide by glucosylceramide synthase (Fig. 1*A*). Glucosylceramide represents the precursor of a large variety of GSLs (23) that are transported in the secretory pathway from the Golgi to the cell surface (11, 24). The physiological functions of GSLs include the regulation of cell adhesion, cell differentiation, and signal transduction (24–26). Dysfunction of GSL degradation is associated with several inherited diseases that are characterized by the accumulation of GSLs in endosomal/lysosomal compartments (27, 28).

Here we sought to analyze the role of GSLs in the proteolytic processing of APP and the generation of Aβ. By using different cell types that express endogenous APP, we demonstrate that depletion of cells from GSLs results in reduced secretion of

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Figs. 1 ("Toxicity of PDMP in HEK293, SH-SY5Y, and HeLa cells"), 2 ("PDMP decreases secretion of both $APP_{S-\alpha}$ and $APP_{S-\beta}$"), and 3 ("Expression of 695, 751, and 770 isoforms of APP in SH-SY5Y cells").

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[1] The abbreviations used are: Aβ, amyloid β-peptide; AD, Alzheimer disease; APP, β-amyloid precursor protein; APP_S, soluble APP; CTF, C-terminal fragment; ECL, enhanced chemiluminescence; GSL, glycosphingolipid; HEK, human embryonic kidney; PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; TRITC, tetramethylrhodamine isothiocyanate; WGA, wheat germ agglutinin.

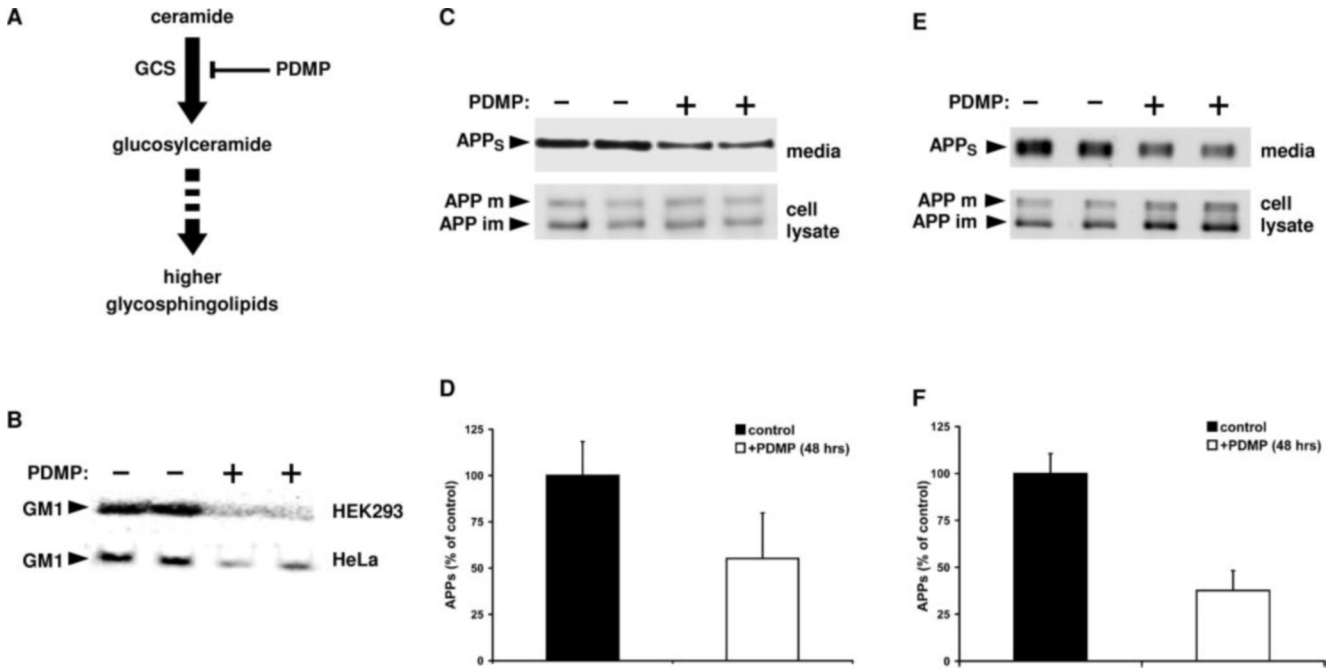

FIG. 1. **Inhibition of GSL biosynthesis decreases the secretion of APP_S.** *A*, schematic showing the biosynthesis pathway of glycosphingolipids and the targeted inhibition of glucosyl ceramide synthase (*GCS*) by PDMP. *B*, detection of GM1. HEK293 (*top*) or HeLa (*bottom*) cells were cultured in the absence (−) or presence (+) of 10 μM PDMP for 48 h. Cellular membranes were separated by SDS-PAGE, and GM1 was detected by Western immunoblotting with cholera toxin. *C*, HEK293 cells were cultured in the absence (−) or presence (+) of 10 μM PDMP for 48 h. APP was immunoprecipitated from conditioned media (*top*) and cell lysates (*bottom*) and separated by SDS-PAGE. Secreted (APP_S) and cellular APP were detected by Western immunoblotting. Migration of APP_S and mature (*m*) and immature (*im*) APP is indicated by *arrowheads*. *D*, secretion of APP_S was quantified by ECL imaging and normalization to cellular APP expression. Values represent the means of three independent experiments ± S.D. (*solid bar*, no PDMP; *open bar*, 10 μM PDMP). *E* and *F*, a similar experiment as that described for *panels C* and *D* was carried out with HeLa cells.

soluble APP and Aβ. Our data indicate that GSLs are implicated in the transport of APP in the secretory pathway and its expression at the cell surface, thereby altering the proteolytic processing by secretases.

MATERIALS AND METHODS

Reagents and Antibodies—D- and L-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), C6-ceramide, and TRITC-labeled wheat germ agglutinin (WGA) were obtained from Sigma. Purified gangliosides from bovine brain were obtained from Fidia Research Laboratories (Abano Therme, Italy). Antibodies 5313 and 6687, recognizing the N- and C-terminal domains of APP, respectively, were described earlier (29) and generously provided by Dr. C. Haass. Polyclonal antibodies 2964 against Aβ were raised by inoculation of rabbits with synthetic Aβ40. Monoclonal antibody 6E10 was obtained from Signet Inc.

Cell Culture and Treatment—B16 and GM95 cells were obtained from the RIKEN cell bank (Tokyo, Japan) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Human embryonic kidney (HEK) 293 and HeLa cells were also cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. SH-SY5Y cells were maintained in RPMI supplemented with 15% fetal calf serum. PDMP was dissolved in water at concentrations of 50 mM and applied to cells as indicated (Figs. 1–6). C6-ceramide was dissolved at a concentration of 10 mM in ethanol. Cells were cultured in the absence or presence of PDMP, ceramide, or exogenous GSLs as indicated (Figs. 1–6). Control cells were incubated with the carrier alone.

Cell Staining with TRITC-labeled WGA—To visualize cell surface glycoproteins, cells grown on coverslips were fixed in 4% paraformaldehyde and incubated with TRITC-labeled WGA (Sigma) for 30 min. Samples were analyzed by fluorescence microscopy on a Nikon Eclipse E800 fluorescence microscope.

Metabolic Labeling, Immunoprecipitation, and Immunoblotting—For pulse-chase experiments, cells were starved at 37 °C in methionine-free, serum-free medium for 45 min and then labeled with [^{35}S]methionine/[^{35}S]cysteine (MP Biomedicals Inc.) at 37 °C for 10 min. Cells were then washed with phosphate-buffered saline and chased in medium supplemented with 10% fetal calf serum and excess amounts of unlabeled methionine for the indicated periods of time. Cells were lysed in STEN buffer (50 mM Tris, pH 7.6, 150 mM NaCl, and 2 mM EDTA) supplemented with 1% Nonidet P-40, 1% Triton X-100, and 2% bovine serum albumin on ice for 10 min. Lysates were clarified by centrifugation for 20 min at 14000 × *g* and immunoprecipitated for 3 h at 4 °C. After separation by SDS-PAGE, proteins were transferred to nitrocellulose membrane (Schleicher & Schuell Inc.) and analyzed by autoradiography or phosphorimaging. Alternatively, proteins were detected by immunoblotting using enhanced chemiluminescence reagent ECL (Amersham Biosciences).

Analysis of Total Protein Secretion—Whatman 3MM paper was cut into squares (2 × 2 cm), soaked with 10% trichloroacetic acid (w/v), and dried. Conditioned chase media (25 μl) of radiolabeled cells was applied to the paper and left to dry. The filter papers were washed in 5% trichloroacetic acid (w/v), absolute ethanol, and acetone (twice each). Subsequent to drying, the filter papers were transferred to scintillation counter vials containing 5 ml of Optiphase Highsafe II scintillation mixture. The activities of ^{35}S in the samples were counted on a scintillation counter using a 60-s time window.

Isolation of Membranes and Detection of GM1—Cells were scraped from the culture dishes and incubated in hypotonic buffer (10 mM Tris, pH 7.3, 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA) for 10 min on ice. Cells were then homogenized by passing 15 times through a 21-gauge needle and centrifuged for 10 min at 1000 rpm to pellet nuclei. The resulting supernatant was centrifuged 30 min at 16000 × *g*. Pelleted membranes were separated by SDS-PAGE, and GM1 was detected by Western immunoblotting with horseradish peroxidase-conjugated cholera toxin (Sigma).

Biotinylation of Cell Surface Proteins—To label cell surface proteins, cells were washed three times with ice-cold phosphate-buffered saline and incubated on ice with phosphate-buffered saline containing 0.5 mg/ml EZ-Link™ sulfo-*N*-hydroxysuccinimide-biotin (Perbio Inc.) for 30 min. Cells were then washed three times with ice-cold phosphate-buffered saline supplemented with 20 mM glycine and finally lysed with STEN buffer containing 1% Nonidet P-40 and 1% Triton X-100. Biotinylated proteins were precipitated from cleared lysates with streptavidin-conjugated agarose beads (Sigma) and separated by SDS-PAGE. The respective proteins were then detected by Western immunoblotting.

Reverse Transcription PCR—Total RNA was isolated from B16 and

GM95 cells using TRIzol, followed by reverse transcription to obtain cDNA. Semi-quantitaive PCR was then performed for APP and actin cDNA with 18 cycles. Primer pairs were 5′-GGTGGACTCTGTGCCAGC-3′ and 5′-TCCGTTCTGCTGCATCTTGG-3′ for APP and 5′-TGCGTGACATCAAAGAGAAG-3′ and 5′-GCTCATAGCTCTTCTCCAGG-3′ for actin.

Data Analysis and Statistics—In metabolic labeling experiments, band intensities were analyzed with a phosphorimaging device (FLA2000; Fuji) and the Fuji Image Gauge 3.0 software. For enhanced chemiluminescence detection, signals were measured and analyzed using an ECL imager (ChemiDoc™ XRS; Bio-Rad) and the Quantity One software package (Bio-Rad). For quantitations, three independent experiments ($n = 3$) were carried out. Statistical analysis was carried out using Student's t test. Significance values are as follows: *, $p < 0.05$; **, $p < 0.01$ (Figs. 3 and 4).

RESULTS

To analyze the role of GSLs in the proteolytic processing of APP and Aβ generation, GSL biosynthesis was inhibited with PDMP, a competitive inhibitor of glucosylceramide synthase that has been shown previously to efficiently decrease GSL biosynthesis in cultured cells (30–33). Treatment of HEK293 or HeLa cells with PDMP for 48 h led to a significant decrease in GSL biosynthesis as demonstrated by a strong reduction of GM1 levels (Fig. 1*B*). The treatment of cells with PDMP at the concentrations used did not affect cell viability (Supplemental Fig. 1, available in the on-line version of this article). We therefore used this approach to investigate the role of GSLs in APP processing. HEK293 or HeLa cells were treated with PDMP, and APP was immunoprecipitated from conditioned media and lysates. As demonstrated in Fig. 1, the secretion of APP_S into conditioned media was markedly decreased in both HEK293 and HeLa cells that were treated with PDMP (Fig. 1, *C–F*). The decrease in APP secretion was observed for both variants APP_S-α and APP_S-β (Supplemental Fig. 2, available in the on-line version of this article). We also assessed the effect of PDMP on the precursor-product relationship of cellular and secreted APP in a pulse-chase experiment. In control cells, ~8% of ^{35}S-labeled cellular APP was secreted into the conditioned media after 2 h. In the same time period, only ~ 2.5% of cellular APP was secreted in PDMP-treated cells (not shown). Together, these data demonstrate that PDMP treatment significantly decreases the secretion of APP. It should be noted that the effects of PDMP on APP secretion were selectively observed in non-transfected cells, but not in cells that stably overexpress APP (data not shown). This might be due to a tight regulation of the interaction of APP with membrane lipids (see "Discussion"). We therefore used exclusively non-transfected cells for the further experiments.

To prove that the effects of PDMP are due to decreased levels of GSLs, we first analyzed the effect of short term treatment with PDMP. In contrast to long term treatment (48 h), incubation of cells with PDMP for only 2 h did not significantly reduce levels of GSLs (data not shown). Under these conditions, the secretion of APP_S was not significantly changed (Fig. 2*A*). We also assessed the effect of the inactive enantiomer L-PDMP that does not inhibit glucosylceramide synthase. Long term treatment of cells for 48 h with L-PDMP did not reduce APP_S secretion (Fig. 2*B*). Together, these control experiments indicate that the reduced secretion of APP_S observed upon cell treatment with PDMP is due to decreased GSL levels in cellular membranes.

We next tested the effect of exogenous GSLs on proteolytic processing of APP. Cells were cultured in the presence or absence of purified bovine brain gangliosides, and levels of cellular and secreted APP were analyzed. The addition of exogenous GSLs significantly increased the cellular levels of endogenous APP as well as the secretion of APP_S (Fig. 2*C*). Together, these data demonstrate a regulatory role of GSLs in the cellular metabolism of APP. The levels of endogenous Aβ in the conditioned media of HEK293 were below the detection limits (data not shown), likely due to efficient cleavage of APP by α-secretase in this cell type (34) (see Supplemental Fig. 2).

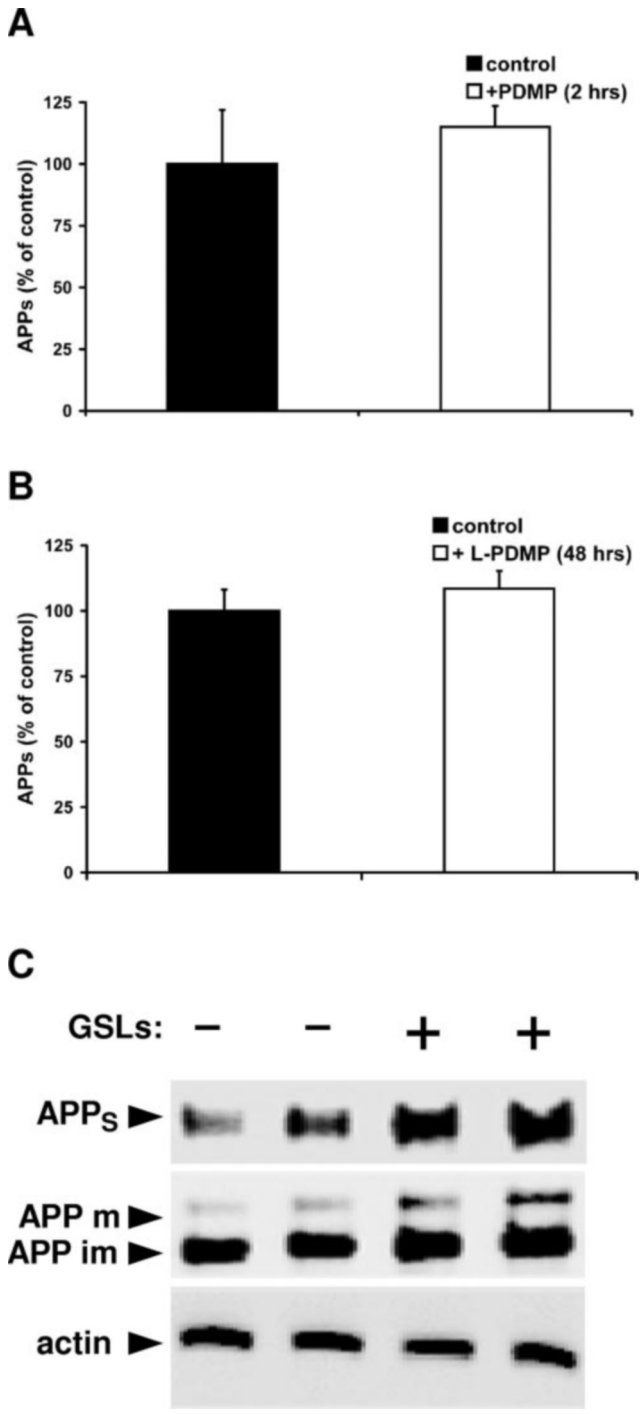

FIG. 2. *A*, HEK293 cells were incubated for 2 h in the absence or presence of 25 μM PDMP, and the secretion of APP_S was analyzed by ECL imaging as described in the legend for Fig. 1. Secretion of vehicle-treated cells was set as 100%. Values represent the means of three independent experiments ± S.D. *B*, cells were cultured for 48 h with the inactive enantiomer L-PDMP, and secretion of APP_S was quantified as in *panel A*. *C*, increased expression and secretion of APP by exogenous gangliosides. HEK293 cells were cultured for 48 h in the absence (−) or presence (+) of 50 μg/ml purified ganglioside mixture from bovine brain. APP_S in conditioned media (*top*) and cellular APP in isolated cell membranes (*middle*) were detected by Western immunoblotting. APP_S and mature (*m*) and immature (*im*) APP are indicated by *arrowheads*. Actin was detected as a loading control (*bottom*).

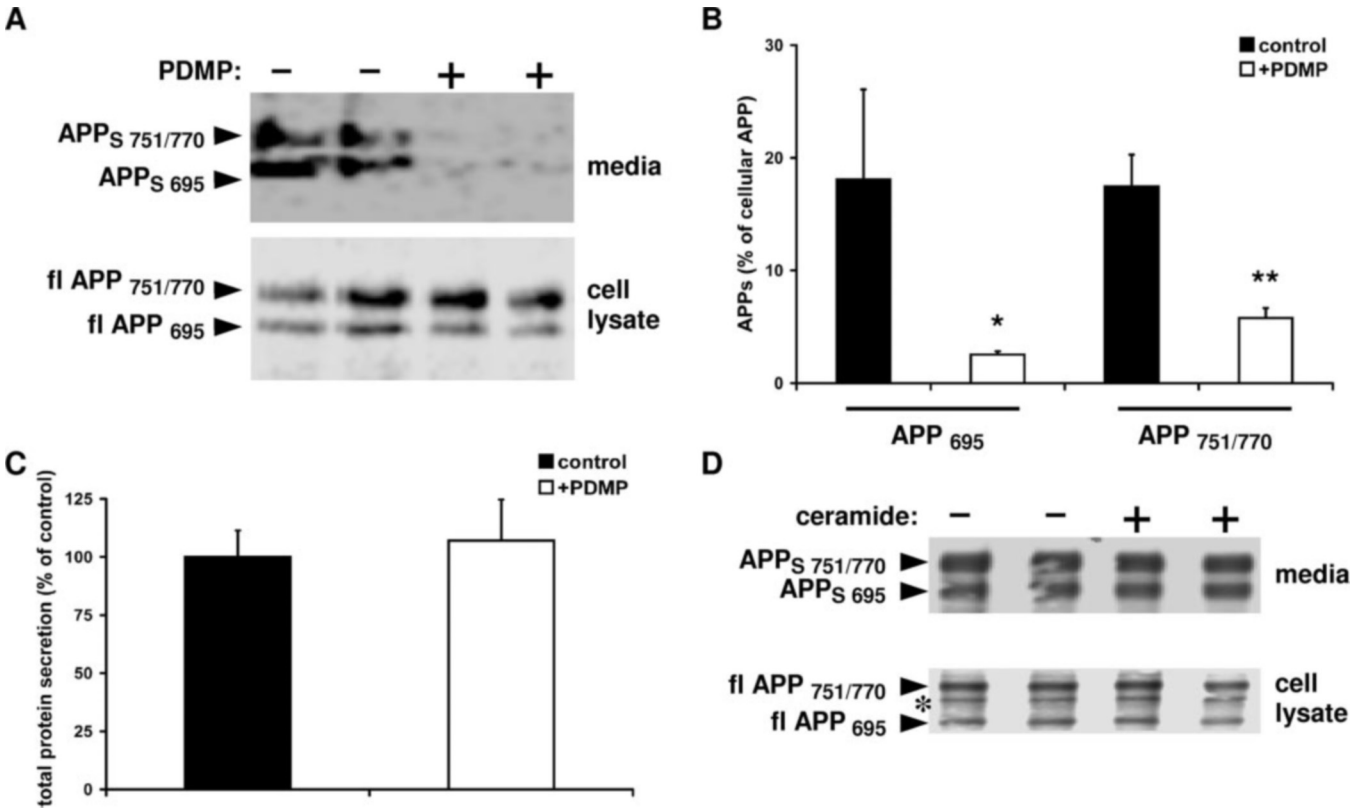

FIG. 3. **GSL depletion selectively inhibits secretion of APP_S in SH-SY5Y cells.** *A*, human SH-SY5Y cells were cultured in the absence (−) or presence (+) of 25 μM PDMP for 48 h and then pulse-labeled with [^{35}S]methionine for 15 min. One set of cells was immediately lysed after the pulse. Another set of cells was incubated for an additional 2 h in the absence or presence of PDMP. APP was immunoprecipitated from the cell lysates (*bottom*) and chase media (*top*) and separated by SDS-PAGE. Radiolabeled APP was detected by phosphorimaging. The migration of APP_S and full-length APP (*fl APP*) for the different splice variants $APP_{751/770}$ and APP_{695} is indicated by *arrowheads*. *B*, quantification of APP secretion was carried out by phosphorimaging. Values represent the means of three independent experiments ± S.D. *C*, total protein secretion was analyzed by trichloroacetic acid precipitation of proteins from the conditioned medium after the chase period. Radioactivity was determined by liquid scintillation counting. Values represent the means of three independent experiments ± S.D. *D*, cells were treated with 10 μM C6-ceramide for 48 h, and APP was immunoprecipitated from conditioned media and cell lysates and detected by Western blotting. The *band* marked by an *asterisk* likely represents mature APP695.

Because neuronal cells secrete higher levels of Aβ, we used human neuroblastoma SH-SY5Y cells to prove a role of GSLs in the processing of APP by pulse-chase experiments. Two variants of endogenously expressed APP were detected after pulse labeling in cell lysates that represent distinct APP splice forms, including the neuron-specific APP_{695} form (Fig. 3*A*). The presence of distinct splice variants in SH-SY5Y cells was also confirmed by reverse transcription PCR using isoform-specific primers (Supplemental Fig. 3, available in the on-line version of this article). PDMP treatment did not affect the expression of the distinct APP variants, as demonstrated by the similar levels of cellular APP after pulse labeling (Fig. 3*A*). Also, no effect on cell viability was detected (Supplemental Fig. 1*C*). As observed in HEK293 and HeLa cells, PDMP resulted in a significant reduction of APP_S secretion into the conditioned media of SH-SY5Y cells (Fig. 3, *A* and *B*). The reduction was observed for the $APP_{751/770}$ as well as for the neuron-specific APP_{695} splice variants. In contrast to HEK293 cells, SH-SY5Y cells predominantly secrete APP_S-β (not shown). Together, these data indicate that PDMP treatment inhibits secretion of both APP_S-α and APP_S-β. However, total protein secretion was not reduced upon PDMP treatment, indicating a selective role of GSLs in the secretion of APP (Fig. 3*C*).

Because the inhibition of glucosylceramide synthase might lead to accumulation of its substrate ceramide, which was shown to alter the proteolytic processing of APP by stabilization of the β-site APP-cleaving enzyme BACE1 (35), we tested the effect of C6-ceramide on the secretion of APP_S. The treatment of cells with ceramide at concentrations of 10 μM, which was shown to stabilize BACE1 (35), did not inhibit the secretion of APP_S, indicating that the inhibition of APP secretion observed after PDMP was due to decreased levels of GSLs (Fig. 3*D*).

To investigate the role of GSLs in the generation of Aβ, SH-SY5Y cells were incubated in the presence or absence of PDMP for 48 h, and Aβ was immunoprecipitated from conditioned media. The secretion of Aβ was significantly reduced upon the inhibition of GSL biosynthesis (Fig. 4, *A* and *B*). On the other hand, the addition of exogenous GSLs increased the secretion of Aβ (Fig. 4, *C* and *D*), indicating that GSLs participate in the generation of Aβ.

We also analyzed the APP CTFs that derive from proteolytic processing of APP by β-secretase or α-secretase. Two major species of APP CTFs were detected that represent CTF-β and CTF-α resulting from β- and α-secretase cleavage, respectively (Fig. 4*E*). In addition, an intermediate band was detected that likely represents CTF-β', a variant generated by an alternative cleavage of APP by β-secretase at Glu-11 within the Aβ domain (36, 37). Consistent with predominant secretion of APP_S-β in this cell type, CTF-β was the predominant species in control cells (Fig. 4, *E* and *F*). The depletion of GSLs resulted in almost similar levels of CTF-β and CTF-α (Fig. 4, *E* and *F*), probably due to further processing of CTF-β by α-secretase (see "Discussion").

It has been shown that proteolytic processing of APP occurs predominantly in post-Golgi secretory and endocytic compartments and at the cell surface (5, 6, 38). We therefore assessed the expression of APP at the cell surface by biotinylation with

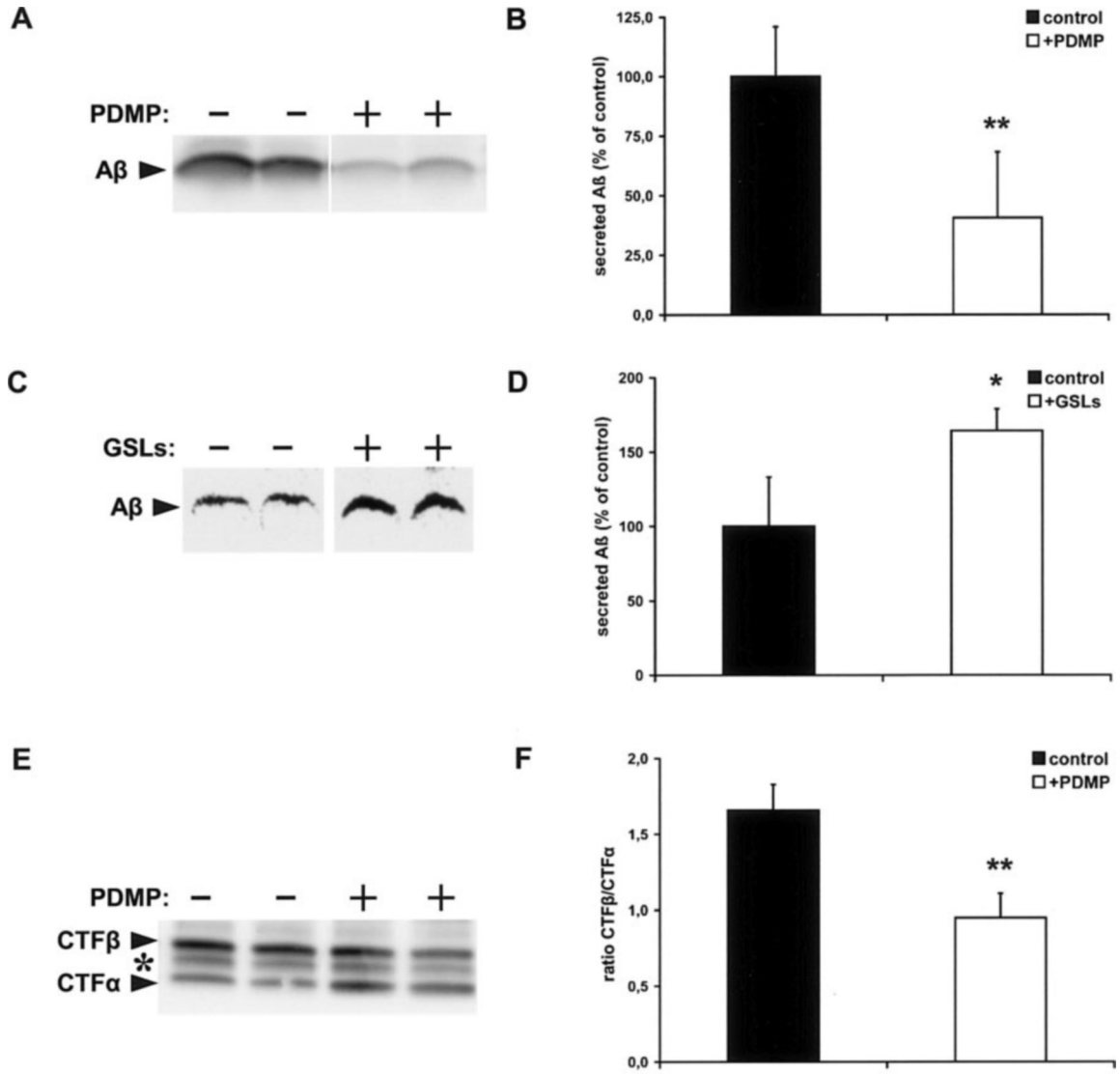

FIG. 4. **GSLs regulate secretion of Aβ.** *A*, human SH-SY5Y cells were cultured in the absence (−) or presence (+) of 25 μM PDMP for 48 h, and endogenously generated Aβ was immunoprecipitated from conditioned media and detected by Western immunoblotting. *B*, Aβ secretion was quantified by ECL imaging and normalized to cellular APP. *C* and *D*, SH-SY5Y cells were cultured in the absence (−) or presence (+) of exogenous GSLs, and the secretion of Aβ was analyzed. *E*, membranes were isolated from PDMP-treated (+) and untreated (−) cells. After separation by SDS-PAGE, CTFs were detected by Western immunoblotting. CTFs generated by β-secretase cleavage (*CTFβ*) and α-secretase cleavage (*CTFα*), respectively, are indicated by *arrowheads*. The CTF generated by alternative β-secretase cleavage is indicated by an *asterisk*. *F*, quantification of the relative amounts of CTFs was done by ECL imaging. Values represent the means of three independent experiments ± S.D.

sulfo-*N*-hydroxysuccinimide-biotin. In SH-SY5Y cells, biotinylated APP could not be detected (not shown), probably due to very efficient proteolytic processing and secretion in this cell type that results in low levels of surface APP. In contrast, biotin-labeled APP could be readily detected in HEK293 cells (Fig. 5*A*). In GSL-depleted cells, the levels of biotin-labeled APP were markedly reduced, demonstrating that suppression of GSL biosynthesis reduces the expression of APP at the cell surface. In contrast, the cell surface expression of the endogenous Fas receptor, also a type I membrane protein, was not decreased upon GSL depletion (Fig. 5*B*). We also assessed the effect of PDMP on the general expression of cell surface proteins by cell staining with TRITC-labeled WGA, a lectin that binds to glycoproteins. No significant difference in the cell surface expression of glycoproteins was observed between treated and non-treated cells (Fig. 5*C*). In addition, the levels of total biotinylated proteins detected by streptavidin-conjugated horseradish peroxidase were very similar in treated and non-treated cells (not shown). Together, these experiments demonstrate that GSL depletion selectively reduced the cell surface expression of APP without generally affecting other membrane proteins. However, the data do not exclude the possibility that cell surface expression of other selected membrane proteins is also affected by GSL depletion.

The decreased expression of APP at the cell surface and reduced secretion of APP_S-α and APP_S-β after GSL depletion suggested that GSLs might be implicated in the forward transport of APP in the secretory pathway. To address this possibility, we performed pulse-chase experiments and analyzed the maturation of APP that occurs in the Golgi compartment. Cells were labeled with [^{35}S]methionine for 10 min and then chased for various time periods. After pulse labeling, a prominent band was detected representing endogenous immature (*N*′-glycosylated) APP. After 30 min of chase, a slower migrating form appeared that represents mature (*N*′/*O*′-glycosylated) APP. The mature form becomes predominant after 60 and 90 min (Fig. 6*A*). GSL depletion reduced the transport of APP to or within the Golgi compartment as indicated by decreased maturation of APP in PDMP-treated cells (Fig. 6, *A* and *B*). We also observed decreased levels of total APP in GSL-depleted cells

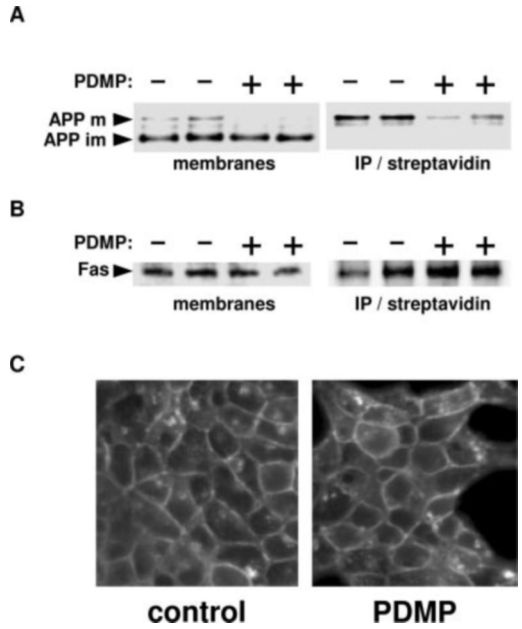

FIG. 5. **GSL depletion decreases expression of APP at the cell surface.** *A* and *B*, cell surface proteins of control (−) and PDMP-treated (+) HEK293 cells were labeled with sulfo-*N*-hydroxysuccinimide-biotin and isolated with streptavidin-conjugated agarose beads as described under "Materials and Methods." Precipitates were separated by SDS-PAGE, and endogenously expressed APP (*A*) or Fas (*B*) was detected by Western immunoblotting (*right sections*). As a control, cellular levels of APP (*panel A*, *left section*) and Fas (*panel B*, *left section*) were also analyzed by Western immunoblotting of isolated cell membranes with the respective antibodies. *IP*, immunoprecipitation. *C*, control (*left*) or PDMP-treated cells (*right*) were stained with TRITC-labeled WGA as described under "Materials and Methods" to detect cell surface-located glycoproteins and analyzed by fluorescence microscopy.

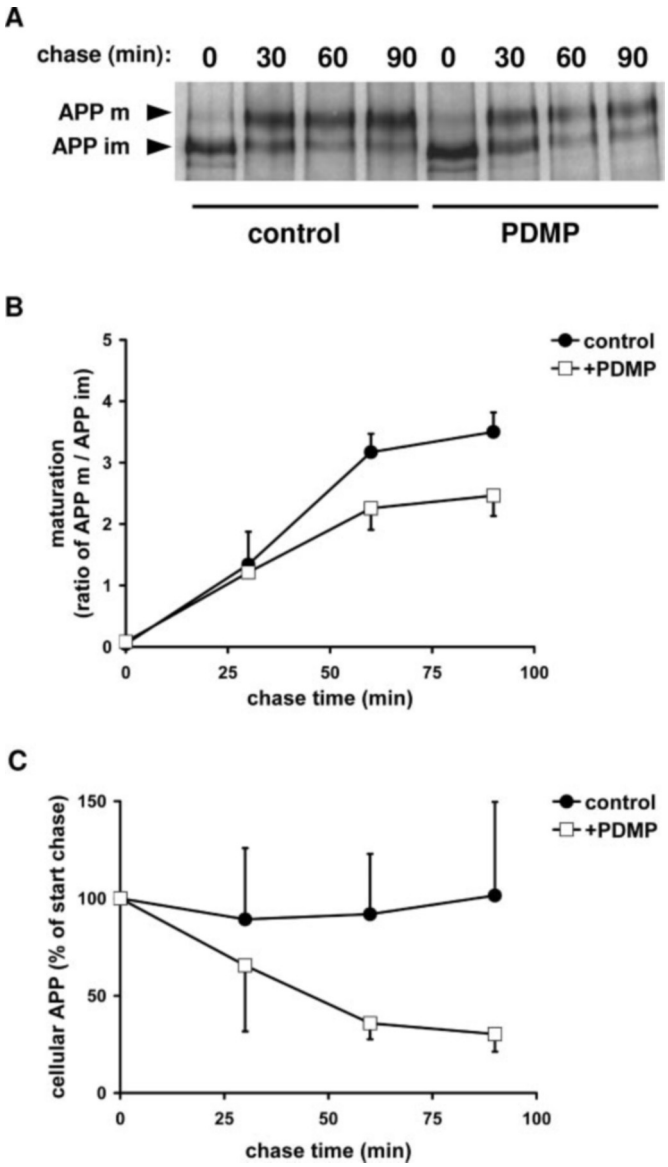

FIG. 6. **Suppression of GSL biosynthesis affects maturation and stability of APP.** *A*, after culturing in the absence or presence of 10 μM PDMP for 48 h, HeLa cells were labeled with [^{35}S]methionine for 10 min and chased for the indicated time periods (see "Materials and Methods"). APP was immunoprecipitated from cell lysates, separated by SDS-PAGE, and detected by phosphorimaging. The migration of mature (*m*) and immature (*im*) APP is indicated by *arrowheads*. *B* and *C*, quantification of APP maturation (*B*) and stability (*C*). In PDMP-treated cells (*open squares*) the maturation of APP is significantly decreased as compared with untreated cells (*closed circles*) (*B*). In addition, the stability of cellular APP is reduced in PDMP-treated cells (*C*). Values represent means of three independent experiments ± S.D.

after chase periods of 60 and 90 min (Fig. 6, *A* and *C*). Because PDMP also reduced the secretion of APP_S (see Figs. 1 and 3), these data indicate an increased degradation of cellular APP in GSL-deficient cells. Indeed, when cells were cultured in the presence of PDMP for 2 weeks we also observed a marked decrease in the levels of mature APP under steady state conditions (data not shown).

To prove these findings in an independent cellular model, we used the mouse melanoma cell lines B16 and GM95. Whereas B16 cells produce GSLs, GM95 cells are defective in GSL biosynthesis due to decreased activity of glucosylceramide synthase and are commonly used as a model of GSL-deficient cells (39–45). As expected, very little if any GM1 could be detected in GM95 cells, whereas B16 cells express robust amounts of GM1 (Fig. 7*A*). To investigate the maturation of APP in both cell types, we performed pulse-chase experiments. In B16 cells, endogenous APP undergoes maturation as indicated by the appearance of a slower migrating band during the chase period (Fig. 7*B*, *left*). In contrast, the GSL-deficient GM95 cell line revealed significantly reduced maturation of APP (Fig. 7*B*, *right*), which is consistent with the data obtained with pharmacological inhibition of GSL biosynthesis (see Fig. 6). In addition, steady state levels of cellular APP were strongly decreased in GM95 cells as compared with B16 cells (Fig. 7*C*), whereas expression of APP mRNA was similar in both cell lines (Fig. 7*D*). Together, these data indicate that GSLs facilitate the maturation and stabilization of APP.

DISCUSSION

We demonstrate that GSLs are implicated in the regulation of proteolytic processing and subcellular transport of APP. The inhibition of GSL biosynthesis reduced the secretion of APP_S and Aβ in different cell types, whereas the addition of exogenous brain gangliosides reversed these effects. As demonstrated by pulse-chase experiments and cell surface biotinylation, the reduction of cellular GSLs decreased the maturation of APP in the secretory pathway and its expression at the cell surface. These effects were observed in cells with pharmacologically or genetically altered GSL biosynthesis. Of note, the effects are observed selectively for endogenous APP, whereas overexpressed APP revealed unaltered maturation and secretion (data not shown). This might be due to tightly regulated interactions of APP with specific lipids within cellular membranes. The overexpression of membrane proteins might therefore lead to altered interactions with lipids, probably by saturation effects (46).

Recent evidence suggests that GSLs are implicated in the

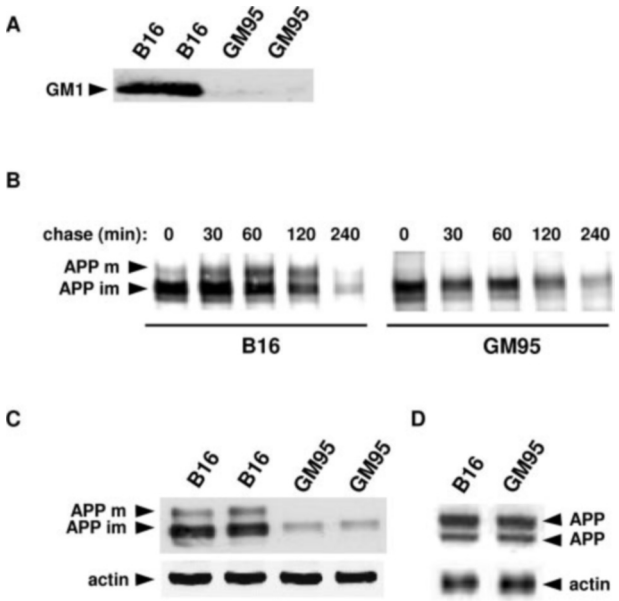

FIG. 7. **Decreased maturation and cellular levels of APP in GSL-deficient cells.** *A*, GM1 in the mouse melanoma cell lines B16 and GM95 was detected by immunoblotting with choleratoxin. *B*, pulse-chase experiments for APP in B16 (*left*) and GM95 (*right*) cells were carried out as described for Fig. 6*A*. The maturation of APP was significantly decreased in GSL-deficient GM95 cells as compared with B16 cells (*m*, mature; *im*, immature). *C*, steady state levels of APP in B16 and GM95 cells were compared by Western immunoblotting. Actin was used as a loading control. *D*, mRNA expression of APP (*top*) was analyzed by reverse transcription PCR as described under "Materials and Methods." Expression of actin mRNA was analyzed as control (*bottom*).

pathogenesis of AD. The levels of individual GSLs are altered in AD brains (19). Moreover, the ganglioside GM1 has been detected in amyloid plaques and binds specifically to Aβ (20, 21). GM1 might also facilitate the aggregation of Aβ into fibrils and deposition in amyloid plaques (22). Interestingly, treatment of APP transgenic mice with GM1 efficiently reduced plaque load in the brains, likely due to sequestering peripheral Aβ (47).

The precise role of GSLs in the proteolytic processing of APP and Aβ production is not well understood. The inhibition of ceramide synthesis in Chinese hamster ovary cells that overexpress human APP resulted in elevated secretion of APP_S, likely due to an increase in α-secretase cleavage (48). Here, we observed a significant decrease in the secretion of endogenous APP in different cell types upon the depletion of GSLs. This discrepancy might be explained by overexpression of APP in the former study (48) that might mask some effects on transport and/or processing of APP (see above). Moreover, in our approach GSL biosynthesis was targeted selectively, whereas the inhibition of ceramide synthesis led also to a strong decrease in the biosynthesis of sphingomyelin that might serve additional functions in APP metabolism (48). In another study, the direct addition of GM1 to cells that overexpress human APP led to increased secretion of Aβ, whereas secretion of APP_S-α was attenuated (49). Because GM1 incorporates into the plasma membrane, these effects might involve alterations in α- and γ-secretase cleavage of APP at the cell surface without affecting transport of APP in the secretory pathway. By targeting GSL biosynthesis, which occurs in the secretory pathway, we could demonstrate that GSLs are also implicated in the maturation of APP in the Golgi and the further transport to the cell surface. However, our data do not rule out the possibility that GSLs might have additional effects on the proteolytic processing of APP at the cell surface, such as direct or indirect modulation of secretase activities (48, 49).

Apart from their role in adhesion processes and signal transduction at the cell surface (24, 50), GSLs have been implicated previously in subcellular protein transport (24, 27, 51). Studies with yeast cells have shown that the inhibition of GSL biosynthesis affects forward transport and stable membrane association of glycosylphosphatidylinositol-anchored proteins (52, 53). Less is known about the role of GSLs in protein transport in the secretory pathway in mammalian cells. Recently, it has been shown that GSLs are involved in the sorting of tyrosinase from Golgi compartments to melanosomes in mouse melanoma cells (40). In agreement with our data, these studies in both yeast and mouse melanocytes also demonstrated that inhibition of GSL biosynthesis does not generally impair protein transport or secretion (40, 52, 53). Thus, GSLs appear to mediate the transport of individual proteins, probably at distinct steps in the secretory pathway. The attenuated transport of APP to the cell surface in GSL-deficient cells is consistent with the decreased secretion of APP by α-secretase, which is known to occur during transport to or directly at the cell surface (54, 55). In contrast, β-secretase cleavage likely occurs predominantly in endocytic compartments after re-internalization of APP from the cell surface (6, 38). Thus, the decreased generation of Aβ upon the depletion of cells from GSLs might be due to decreased access of β-secretase to APP in endocytic compartments. The decreased levels of CTF-β in GSL-depleted SH-SY5Y cells are in agreement with this notion. Taken together, our data indicate that GSLs and the respective enzymes involved in their biosynthesis might represent targets to decrease formation of Aβ in therapeutic strategies for AD.

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