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ABSTRACT

Presenilins (PSs) are components of the gamma-secretase complex that mediates intramembranous cleavage of type I membrane proteins. We show that gamma-secretase is involved in the regulation of cellular lipoprotein uptake. Loss of gamma-secretase function decreased endocytosis of low-density lipoprotein (LDL) receptor. The decreased uptake of lipoproteins led to upregulation of cellular cholesterol biosynthesis by increased expression of CYP51 and enhanced metabolism of lanosterol. Genetic deletion of PS1 or transgenic expression of PS1 mutants that cause early-onset Alzheimer's disease led to accumulation of gamma-secretase substrates and mistargeting of adaptor proteins that regulate endocytosis of the LDL receptor. Consistent with decreased endocytosis of these receptors, PS1 mutant mice have elevated levels of apolipoprotein E in the brain. Thus, these data demonstrate a functional link between two major genetic factors that cause early-onset and late-onset Alzheimer's disease.

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Loss of \( \gamma \)-Secretase Function Impairs Endocytosis of Lipoprotein Particles and Membrane Cholesterol Homeostasis

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Presenilins (PSs) are components of the \( \gamma \)-secretase complex that mediates intramembranous cleavage of type I membrane proteins. We show that \( \gamma \)-secretase is involved in the regulation of cellular lipoprotein uptake. Loss of \( \gamma \)-secretase function decreased endocytosis of low-density lipoprotein (LDL) receptor. The decreased uptake of lipoproteins led to upregulation of cellular cholesterol biosynthesis by increased expression of CYP51 and enhanced metabolism of lanosterol. Genetic deletion of PS1 or transgenic expression of PS1 mutants that cause early-onset Alzheimer’s disease led to accumulation of \( \gamma \)-secretase substrates and mistargeting of adaptor proteins that regulate endocytosis of the LDL receptor. Consistent with decreased endocytosis of these receptors, PS1 mutant mice have elevated levels of apolipoprotein E in the brain. Thus, these data demonstrate a functional link between two major genetic factors that cause early-onset and late-onset Alzheimer’s disease.

Key words: presenilin; lipoprotein uptake; apo E; SREBP2; cholesterol; APP

Introduction

Cellular membrane cholesterol levels are controlled by \textit{de novo} synthesis, storage in form of cholesterol esters, and the uptake and release of cholesterol in complexes with lipoproteins (Brown and Goldstein, 1997). Low cellular cholesterol levels induce biosynthesis by complex transcriptional and posttranslational mechanisms involving proteolytic processing of sterol regulatory element binding proteins (SREBPs) to increase expression of enzymes involved in cholesterol synthesis. Higher cholesterol levels inhibit the proteolytic processing of SREBPs and thereby decrease cellular cholesterol biosynthesis (Goldstein et al., 2006).

The internalization of extracellular cholesterol/lipoprotein complexes by receptor-mediated endocytosis involves adaptor proteins that bind to specific amino acid motifs in their cytoplasmic domains (Herz and Bock, 2002; Jeon and Blacklow, 2005). In the human brain, cholesterol is mainly transported in high-density lipoprotein particles that predominantly contain the apolipoprotein E (apo E). Notably, apo E has been linked to Alzheimer’s disease (AD), implicating that altered transport and metabolism of lipids in the brain could contribute to neurodegeneration (Kennedy et al., 2003; Tanzi and Bertram, 2005).

Whereas the apo E4 allele is a major risk factor for late-onset AD, mutations in the presenilin (PS) genes are a major cause of early-onset familial AD (FAD) (Kennedy et al., 2003; Tanzi and Bertram, 2005). PSs are the catalytic components of \( \gamma \)-secretase complexes that mediate cleavage of type I membrane proteins (Selkoe and Kopan, 2003; Parks and Curtis, 2007). The cleavage of the \( \beta \)-amyloid precursor protein (APP) by \( \gamma \)-secretase results in generation of the amyloid \( \beta \)-peptide (A\( \beta \)), which accumulates in extracellular deposits during the pathogenesis of AD (Selkoe, 2001). In addition to APP, a growing number of additional substrates for \( \gamma \)-secretase have been identified (Selkoe and Wolfe, 2007; Spasic and Annaert, 2008). The cleavage of these proteins is apparently regulated by preceding shedding of their ectodomains by other proteases resulting in the generation of membrane-tethered C-terminal fragments (CTFs) (Struhl and Adachi, 2000). The subsequent intramembranous cleavage of these CTFs by \( \gamma \)-secretase results in the release of intracellular domains.
(ICDs) from cellular membranes that can serve signaling functions. This process is well understood for Notch signaling where the Notch ICD (NICD) translocates to the nucleus to regulate gene transcription (Sisodia and George-Hyslop, 2002; Selkoe and Kopal, 2003). The importance of this cleavage is demonstrated by the phenotypes of PS knockout animals that closely resemble that of Notch deficiency (Shen et al., 1997; Wong et al., 1997; Herrenman et al., 2000). However, it is far from being clear whether PS-dependent cleavage of other γ-secretase substrates also serve signaling functions or is also important for more general degradation of membrane proteins (Kopan and Ilagan, 2004; Selkoe and Wolfe, 2007). PS proteins also affect the subcellular trafficking of individual membrane proteins, including APP, TrkA, telecephalin, tyrosinase, and transferrin, but the underlying molecular mechanisms remain unclear (Naruse et al., 1998; An et al., 2004).

Here, we show that the accumulation of γ-secretase substrates after partial loss of γ-secretase function impairs endocytosis of lipoproteins. Importantly, FAD-associated mutations of PS1 also induced aberrant endocytosis of apoE.

Materials and Methods
cDNA constructs, antibodies, and reagents. The constructs encoding APP C99-EGFP (Kaether et al., 2006), Fe65-myc (Pietrzik et al., 2004), and APPsA-KI with APPSα-/APLP2-deficient animals. We used APPSα+/APLP2+/-/ interbreeding to obtain littersmates of APLP2+/+-/ and APLP2+/APLP2+/--/ mice, which were then analyzed. APP/APLP1/APLP2 triple knock-out mice were originally described by Herms et al. (2004).

Human brain material was obtained from the University Hospital of Bonn (Bonn, Germany), and the Municipal Hospital of Offenbach (Offenbach, Germany). Detailed description of the brains is in the supplemental material (available at www.jneurosci.org).

Immunohistochemistry and protein analysis of human and mouse brain. Mouse brains were immersion fixed in 4% paraformaldehyde. Blocks of the right hemisphere were embedded in paraffin and microtomed into 5 μm sections. Apo E staining was performed as described earlier (Thal et al., 2005). Homogenization of human and mouse brain protein extraction and detection by Western immunoblotting was performed as described previously (Dewachter et al., 2002; Wahle et al., 2006).

Immunocytochemistry. Cells were grown on polylysine-coated glass coverslips and fixed in 4% paraformaldehyde before processing for immunofluorescence as described previously (Walter et al., 2001). To visualize endocytosis of LDL, 10 μg/ml BODIPY-labeled LDL (Molecular Probes) was added to living cells for 10 min at 37°C in DMEM, and cells were fixed after washing. Endocytosis was monitored by fluorescence microscopy.

Reverse transcription (RT) PCR. For the semiquantitative PCR from MEF cells, total RNA was obtained from WT and dKO cells using Trizol (Invitrogen) followed by cDNA synthesis using oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen). PCR (18 cycles) was performed for the respective cDNAs using the following primer pairs: β-actin: 5′-GCCGCTGACGATCAAGAAGAG-3′ and 5′-GCCATGATCTCTTCCAGG-3′; lanosterol synthase: 5′-AGGAAGCGAGAGGCCGAT-3′ and 5′-TGATTCCTGTTCTCTGAGC-3′; CYP51: 5′-CTGGAACAGACAATCTCT-3′ and 5′-CACACACCTATGTCCTGG-3′; seladin-1: 5′-GAGACACTACTACCCGCAGC-3′ and 5′-TGCCTCGACTGGCTGC-3′.

For quantitative real-time PCR, mouse brain hemispheres were fixed...
in "RNAlater" overnight at −20°C. The other hemisphere was snap frozen and kept at −20°C for biochemical analyses. Total mRNA was prepared from the dissected frontal cortex using the RNeasy purification kit, and cDNA was prepared from 2 μg of total RNA using Applied Biosystems High Capacity cDNA kit. Real-time PCR was performed using SYBR Green (Applied Biosystems) in 96-well plates with 3% aqueous solution of H2O. The PCR was performed in a final volume of 50 μl of 2× SYBR Green Master Mix (Applied Biosystems). Each sample was run in triplicate in one PCR plate. The ABI Prism 7500 Sequence Detection System (Applied Biosystems) was used for the amplification and the determination of the PCR efficiency. The efficiency of the PCR was determined by qPCR analysis of the standard curve. The mRNA levels of the target gene were normalized to the mRNA levels of the reference gene (β-actin). The data were analyzed using the 2−ΔΔCT method.

Biotinylation and endocytosis of lipoprotein receptors. Labeling of cell surface protein has been described earlier (Tamboli et al., 2005). Briefly, cells were incubated on ice with PBS containing 0.5 mg/ml EZ-link sulfo-NHS-biotin (Pierce) for 30 min. Cells were then washed three times with ice-cold PBS supplemented with 20 mM glycine and finally lysed with STEIN buffer containing 1% NP-40/1% Triton X-100. Biotinylated proteins were precipitated from cleaved lysates with streptavidin-conjugated agarose beads (Sigma) and separated by SDS-PAGE. LDL receptor was then detected by Western immunoblotting. To study LDLR endocytosis, cleavable EZ-link sulfo-NHS-SS-biotin was used (Pierce). After biotinylation, cells were further incubated at 37°C for 15 min to allow endocytosis. Endocytosed LDLR was then analyzed by cleavage of biotin from nonendocytosed LDLR using reducing buffer.

Analysis of cellular sterols. Lipids were extracted with chloroform/methanol (2:1; v/v) from cultured cells and dried to constant weight in a Speedvac (Servant Instruments). 5α-Cholestane (Serva Electrophoresis), epiretinsolan (Sigma), and racemic [23,23,24,23–H4] 24(Δ5,7)-OH chol (Medical Isotopes) were added as internal standards. After saponification, extraction, and derivatization, sterols were determined as trimethylsilyl-ethers by using gas-liquid chromatography-flame ionization detection (GC-FID) to analyze cholesterol, and GC-mass spectrometry (GC-MS) to measure concentrations of lanosterol, desmosterol, and cholesterol as described previously (Paivi et al., 2005).

Data analysis and statistics. ECL signals were measured and analyzed using an ECL imager (ChemiDoc XRS, Bio-Rad) and the Quantity One software package (Bio-Rad). For quantitation of cellular sterol concentrations, five independent experiments (n = 5) were carried out. Statistical analysis was done using Student’s t test.

Results

Increased cholesterol biosynthesis in PS-deficient cells via transcriptional upregulation of CYP51

To assess the involvement of γ-secretase in cellular cholesterol metabolism, we performed a comprehensive analysis of cellular sterols in mouse embryonic fibroblasts (MEFs) of WT and PS1/PS2 double knock-out (PS dKO) mice. PS dKO cells showed increased levels of cholesterol compared with WT MEFs (Fig. 1A). Stable overexpression of human PS1 (hPS1) in PS dKO cells (see supplemental Fig. 2, available at www.jneurosci.org as supplemental material) partially normalized cholesterol levels to that of WT cells. In addition, the pharmacological inhibition of endogenous γ-secretase activity in WT MEFs with N-[N-(3,5-difluorophenyl)glycyl]-l-phenylglycine t-butyl ester (DAPT) also led to a significant increase in cholesterol concentrations (Fig. 1A). These data are consistent with previous studies that also indicated an implication of γ-secretase in the regulation of cellular cholesterol levels (Grimm et al., 2005; Liu et al., 2007).

To further analyze whether the increased cholesterol levels in PS-deficient cells were associated with increased de novo synthesis, we determined the levels of desmosterol, an immediate precursor of cholesterol. Desmosterol concentrations were also increased in the PS dKO cells by ~40%. As observed for cholesterol, transfection of PS dKO cells with hPS1 also decreased levels of desmosterol, whereas treatment of WT cells with DAPT led to increased levels of desmosterol (Fig. 1B). Cellular levels of cholesterol, a reduction product of cholesterol, and of the hydroxylated derivative 27-OH-cholesterol were also increased in PS-deficient cells (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), indicating that the elevated levels of cholesterol in PS dKO cells are not caused by impaired downstream metabolism of cholesterol.

The first cyclic metabolite in cholesterol biosynthesis is lanosterol that is generated by cyclization of squalene (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).
The analysis of lanosterol revealed decreased levels of this precursor in PS dKO cells compared with WT cells (Fig. 1C). DAPT treatment of WT MEFs also resulted in decreased lanosterol levels (Fig. 1C). The strongly altered levels of lanosterol led us to analyze the mRNA expression of the three enzymes directly involved in lanosterol metabolism. mRNA levels of lanosterol synthase and seladin-1 were not significantly altered in PS dKO cells compared with WT cells (Fig. 1D). In contrast, mRNA expression of CYP51 was markedly increased in PS dKO cells, indicating that PS deficiency led to increased cholesterol biosynthesis by upregulation of CYP51 expression, and increased metabolism of lanosterol. To prove whether the increased cholesterol biosynthesis in PS-deficient cells could be reversed by inhibition of CYP51 activity, we tested the effect of itraconazole, a selective inhibitor of CYP51 activity, on lanosterol. To prove whether the increased cholesterol biosynthesis in PS-deficient cells could be reversed by inhibition of CYP51 activity, we tested the effect of itraconazole, a selective inhibitor of CYP51 (Païvi et al., 2005). The treatment with itraconazole increased levels of lanosterol in PS dKO cells, proving the inhibition of CYP51 (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). We also observed a subtle but highly significant decrease in cholesterol and desmosterol after itraconazole treatment (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). In contrast, altered sterol levels in PS dKO cells were not normalized by the addition of exogenous Aβ (supplemental Fig. 5, available at www.jneurosci.org as supplemental material).

**Figure 3.** γ-Secretase-dependent distribution and endocytosis of the LDL receptor. **A**, Presenilin-dependent subcellular localization of LDLR was analyzed by immunocytochemistry. Prominent localization of LDLR at the cell surface in PS dKO cells is indicated by arrowheads. PS dKO cells have altered size and morphology compared with WT cells. These alterations were reverted by reexpression of hPS1. Scale bar, 25 μm. **B**, Cell surface proteins were labeled with sulfo-NHS-biotin and precipitated with streptavidin-conjugated agarose. Precipitated LDLR was detected by Western immunoblotting. **C**, Decreased endocytosis of LDLR in PS-deficient cells. Surface proteins of MEF WT and PS dKO cells were labeled with sulfo-NHS-SS-biotin. Cells were incubated for the indicated time periods at 37°C to allow endocytosis, and residual biotin from cell surface was removed using reducing buffer. Internalized biotin-labeled proteins were precipitated and LDLR was detected by Western immunoblotting (see Materials and Methods section for details). **D**, LDLR endocytosis analysis in HEK 293 cells expressing P51 WT or a dominant-negative (DN) variant of P51.

**Loss of γ-secretase decreases lipoprotein uptake**

The expression of CYP51 in endothelial cells is suppressed by the uptake of extracellular LDL (Rodríguez et al., 2001). Because we also observed a significant down-regulation of CYP51 expression after incubation of WT MEFs with exogenous LDL (Fig. 2A), we speculated that the increased expression of CYP51 in PS dKO cells might be caused by impaired uptake of extracellular LDL from the cell culture media. WT cells efficiently internalized BODIPY-labeled LDL into cytoplasmic vesicular structures, demonstrating endocytosis of LDL (Fig. 2B). In contrast, very little internalization of BODIPY-labeled LDL was observed in PS dKO cells, indicating impaired endocytosis of LDL (Fig. 2B). To prove this, we analyzed total cellular levels of LDL in PS WT and dKO cells by Western immunoblotting with an antibody against apolipoprotein B100 (apo B100), a major component of LDL particles in culture serum. Cellular levels of apo B100 were significantly lower in PS dKO cells, also indicating decreased internalization of LDL in PS-deficient cells (Fig. 2C). Consistent with decreased endocytosis of lipoproteins, the cellular levels of sitosterol, an exogenous plant sterol that is associated and internalized with serum lipoproteins, but not metabolized by mammalian cells, were also significantly reduced in PS dKO cells (3.5 ± 0.2 ng/mg dry cell mass in PS dKO (n = 5) versus 14.6 ± 0.5 mg/nm dry cell mass in PS WT cells, p < 0.001). Collectively these data demonstrate an impaired internalization of LDL particles in PS-deficient cells.

Decreased uptake of extracellular LDL should also increase expression of SREBP-2, which itself regulates the expression of CYP51 (Rodríguez et al., 2001). In line with the observed inhibition of LDL uptake, levels of full-length SREBP-2 and its N-terminal fragment that regulates transcription of target genes were significantly increased in PS dKO cells (Fig. 2D).

**Figure 3.** γ-Secretase-dependent distribution and endocytosis of the LDL receptor. **A**, Presenilin-dependent subcellular localization of LDLR was analyzed by immunocytochemistry. Prominent localization of LDLR at the cell surface in PS dKO cells is indicated by arrowheads. PS dKO cells have altered size and morphology compared with WT cells. These alterations were reverted by reexpression of hPS1. Scale bar, 25 μm. **B**, Cell surface proteins were labeled with sulfo-NHS-biotin and precipitated with streptavidin-conjugated agarose. Precipitated LDLR was detected by Western immunoblotting. **C**, Decreased endocytosis of LDLR in PS-deficient cells. Surface proteins of MEF WT and PS dKO cells were labeled with sulfo-NHS-SS-biotin. Cells were incubated for the indicated time periods at 37°C to allow endocytosis, and residual biotin from cell surface was removed using reducing buffer. Internalized biotin-labeled proteins were precipitated and LDLR was detected by Western immunoblotting (see Materials and Methods section for details). **D**, LDLR endocytosis analysis in HEK 293 cells expressing P51 WT or a dominant-negative (DN) variant of P51.
HMG-CoA reductase, another target gene, which is upregulated by SREBP-2, were also increased in PS dKO cells (Fig. 2E). However, levels of HMG-CoA reductase could be also regulated post-translationally by lanosterol levels (Goldstein et al., 2006). Indeed, addition of exogenous lanosterol significantly decreased HMG-CoA reductase levels in PS dKO cells (Fig. 2F). Thus, the increase in HMG-CoA reductase levels could also involve stabilization of the protein by decreased lanosterol concentrations in PS dKO cells. Together these data indicate that inhibition of γ-secretase decreased lipoprotein uptake, which in turn led to upregulation of cholesterol biosynthetic enzymes.

Inhibition of γ-secretase impairs endocytosis of lipoprotein receptors

We next analyzed the expression of the LDL receptor (LDLR) in WT and PS dKO MEFs. Although LDLR was localized predominantly in juxtanuclear structures in WT cells, PS dKO cells showed also prominent localization of the receptor at the plasma membrane. Reexpression of hPS1 in PS dKO cells decreased the localization of LDLR at the cell surface and led to predominant localization in juxtanuclear compartments, very similar to WT cells (Fig. 3A). PS dKO cells also appeared to be enlarged compared with WT cells, and reexpression of hPS1 normalized cell size (Fig. 3A). As revealed by cell-surface labeling with biotin, expression of LDLR at the plasma membrane was increased in PS-deficient cells (Fig. 3B).

The increased expression of LDLR at the cell surface but decreased uptake of LDL after inhibition of γ-secretase suggested impaired endocytosis of LDLR. The analysis of LDLR endocytosis indeed revealed reduced internalization from the plasma membrane in PS-deficient cells (Fig. 3C). We also compared the LDLR endocytosis in HEK293 cells stably overexpressing WT or a DN mutant of PS1. LDLR endocytosis was found to be reduced in cells expressing PS1-DN compared with the cells which express PS1 WT (Fig. 3D). These results indicate that loss of γ-secretase activity rather than loss of PS proteins causes an inhibition of LDLR endocytosis in PS-deficient cells.

Increased apolipoprotein E in brain of mice with neuron-specific deletion of PS1

To prove a role of γ-secretase in lipoprotein metabolism in vivo, we analyzed apo E levels in brain homogenates of mice with a conditional deletion of PS1 in brain neurons (PS1n<sup>−/−</sup>) and control mice (Dewachter et al., 2002). Brain apo E levels were significantly increased in the PS1n<sup>−/−</sup> mice compared with controls, suggesting an involvement of γ-secretase in the metabolism of apo E (Fig. 4A). Immunohistochemical analysis revealed that neuronal deficiency of PS1 also resulted in altered localization of apo E. In control mice, apo E was homogenously distributed in the cytoplasm of cortical neurons, indicating efficient internalization. In contrast, neurons of PS1n<sup>−/−</sup> mice showed decreased cytoplasmic apo E staining, but prominent reactivity at the plasma membrane (Fig. 4B, C). These data indicate that the uptake of apo E is impaired in PS1-deficient neurons in vivo.

Recently, it was reported that the APP intracellular domain (AICD) could regulate the expression of the LDL receptor related protein 1 (LRP1), and increased levels of LRP1 mRNA and protein were found in brains of APP-deficient mice (Liu et al., 2007). Because PS deficiency would lead to decreased AICD production, the lack of AICD after inactivation of γ-secretase could affect the expression of LRP1. We therefore compared LRP1 mRNA expression in brain cortices of embryonic WT mice with that of mice lacking APP and APP-like proteins (APLP1<sup>−/−</sup>, APLP2<sup>−/−</sup>, APLP1<sup>−/−</sup>/APLP2<sup>−/−</sup>, double knock-out; TKO, APP<sup>−/−</sup>/APLP2<sup>−/−</sup>/APLP2<sup>−/−</sup>, triple knock-out). In contrast to previous data (Liu et al., 2007), levels of LRP1 mRNA were not increased after deletion of APP and APLPs (Fig. 5A). Rather, there was a trend to decreased expression of LRP1 mRNA, suggesting that AICD does not suppress transcription of LRP1. In line with this, LRP1 mRNA expression was not increased in embryonic brain cortices.
of PS1 KO mice (data not shown). To test a potential role of AICD in adult mice, we compared the levels of LRP1 mRNA and protein in brains of 22–26 weeks old WT mice with that of age-matched mice deficient in APP (APP−/−) or APLP2 (APLP2−/−). Because APP−/−/APLP2−/− double knock-out mice show early postnatal death (Heber et al., 2000), we also generated mice that express soluble APP (APPsα) in a APLP2−/− background by crossing APPsα knock-in mice with APLP2−/− mice. The resulting APPsα/APLP2−/− (DKI) mice lack the intracellular domains of APP and APLP2, but express the soluble ectodomain of APP. Notably, APPsα/APLP2−/− mice escape early postnatal death, indicating an important physiological function of the APP ectodomain. Whereas LRP1 mRNA was not significantly altered in brains of APLP2−/− and APPsα/APLP2−/− mice, a slight (1.75-fold) increase in brains of adult APP−/− mice was detected (Fig. 5B). These data indicate that lack of AICD does not increase the mRNA expression of LRP1. A potential role of the APP ectodomain in this process remains to be determined in the further studies.

Western immunoblot analysis demonstrated that expression of LRP1 was not increased in the brains of adult APP−/−, APLP2−/−, or APPsα/APLP2−/− mice compared with WT mice (Fig. 5C,D). Importantly, brain levels of apo E were also not altered in the different models, even in the APPsα/APLP2−/− mice that lack ICDs of APP and APLP2 (Fig. 5C–E). Together, these data indicate that lack of AICD is not responsible for the increased levels of apo E observed in the brains of PS1n−/− mice (see Fig. 4).

Increased apo E in mouse and human brain with PS1 FAD mutations
We next tested the effect of FAD-associated PS1 mutations on apo E expression in the brain of transgenic mice. Of note, mice were used that do not overexpress human APP and therefore do not develop deposits of Aβ (Dewachter et al., 2002). Western immunoblotting revealed significantly elevated levels of apo E in brain lysates of PS1 A246E (Fig. 6A). Very similar data were also obtained with mice expressing the PS1 A9 mutation (Fig. 6B). To verify these findings in humans, we compared the expression of apo E in brains of FAD cases harboring mutations in the PS1 gene to that of controls. Apo E levels were markedly increased in brain lysates of FAD patients compared to controls (Fig. 6C,D).

Accumulation of γ-secretase substrates reduces endocytosis of LDLR
Because our data so far did not support an involvement of cleavage products that derive from proteolytic processing of APP by γ-secretase in the regulation of LDLR endocytosis, we tested the role of APP CTFs, the immediate γ-secretase substrate. Consistent with previous studies (De Strooper et al., 1998; Wolfe et al., 1999; De Strooper et al., 1999b), we observed a strong accumulation of APP CTFs in PS-deficient cells and after inhibition of γ-secretase activity by DAPT (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). Importantly, APP CTFs accumulated at the cell surface and in cytoplasmic vesicles after inhibition of γ-secretase with DAPT or in PS-deficient cells. Similar patterns were also observed for the γ-secretase substrates LRP1-CT and NΔE (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). Consistent with earlier studies (Bentahir et al., 2006; Kumar-Singh et al., 2006) we also
observed increased levels of APP-CTFs in cells expressing different PS1 FAD mutants (supplemental Fig. 7, available at www.jneurosci.org as supplemental material), indicating accumulation of γ-secretase substrates as a result of partial loss of proteolytic function by mutations in PS proteins.

Because the membrane-tethered CTFs of several γ-secretase substrates contain amino acid motifs that interact with adaptor proteins involved in the endocytosis of cell surface receptors, we conjectured that impaired cleavage of these substrates could affect their targeting and metabolism. Notably, the adaptor proteins Fe65 and autosomal recessive hypercholesterolemia (ARH), which mediate endocytosis of lipoprotein receptors, also interact with the cytoplasmic domain of APP (Trommsdorff et al., 1998; Herz and Bock, 2002). We therefore tested whether accumulation of APP CTFs interferes with the localization of these adaptor proteins at the plasma membrane. In control cells, Fe65 showed prominent localization in the nucleus (Fig. 7A). However, Fe65 was predominantly targeted to juxtanuclear structures after overexpression of APP CTF (C99-EGFP), indicating an interaction of both proteins in these compartments (Fig. 7B). The pharmacological inhibition of γ-secretase also led to prominent targeting of Fe65 to the plasma membrane together with C99, further indicating aberrant targeting of adaptor proteins as a result of accumulated APP CTFs (Fig. 7B). The localization of ARH was also affected by overexpression of C99-EGFP and subsequent inhibition of γ-secretase. Whereas ARH showed a broader distribution in cells without C99-EGFP (Fig. 7C), the simultaneous expression of C99-EGFP targeted ARH predominantly to juxtanuclear structures (Fig. 7D). Similar to Fe65, inhibition of γ-secretase also led to prominent targeting of ARH to the plasma membrane together with C99-EGFP (Fig. 7D).

To prove that accumulation of APP CTFs could impair the function of LDLR, we analyzed receptor endocytosis by biotinylation experiments. The expression of APP-C99 markedly reduced the internalization of LDLR from the cell surface (Fig. 7E), indicating that accumulation of APP CTFs interferes with the subcellular transport of the receptor. Together, these results are consistent with impaired lipoprotein endocytosis by accumulation of APP CTFs after total or partial loss of γ-secretase function.

**Discussion**

Here, we demonstrate that γ-secretase activity is intimately related to the endocytosis of lipoproteins. The deficiency of PS proteins or FAD-associated mutations decreased the capacity of cells to internalize extracellular lipoproteins by impairment of lipoprotein receptor endocytosis. The comprehensive analysis of cellular sterols and expression of cholesterol biosynthetic enzymes allowed the demonstration of enhanced cholesterol biosynthesis in PS-deficient cells. These data are consistent with previous studies showing that lowering extracellular LDL concentration results in upregulation of cellular cholesterol biosynthesis by increasing expression of HMG-CoA reductase (Brown and Goldstein, 1976). In turn, addition of exogenous LDL to porcine vascular endothelial cells led to suppression of CYP51 mRNA expression via an SREBP-2-dependent pathway (Rodriguez et al., 2001). We also observed increased levels of HMG-CoA reductase and CYP51 in PS-deficient cells, suggesting that the decreased uptake of extracellular LDL led to transcriptional upregulation of these enzymes. Because HMG-CoA reductase is also regulated at the posttranslational level by lanosterol (Goldstein et al., 2006), the decreased concentrations of lanosterol in PS-deficient cells could also stabilize HMG-CoA reductase.

Because Aβ inhibits HMG-CoA reductase activity in vitro, the lack of Aβ could also increase cholesterol levels in PS-deficient cells (Grimm et al., 2005). However, the addition of exogenous Aβ did not normalize the elevated cholesterol levels nor the decreased lanosterol levels of PS-deficient cells. It will therefore be interesting to further dissect the relative contribution of Aβ in cellular cholesterol metabolism.

In line with the impaired lipoprotein uptake, conditional deletion of neuronal PS1 led to increased levels of apo E in mouse brain. Because apo E is mainly synthesized by glial cells that were not targeted by deletion of PS1 in neurons (Dewachter et al., 2002), these data suggest that inefficient clearance of apo E by neuronal cells contributes to increased apo E levels. Whether apo E is also altered at the transcriptional level in these models remains to be determined. Importantly, PS1 FAD mutations also led to increased levels of apo E in mouse brain. Because apo E
together with lipoprotein receptors also mediate A/β clearance within the brain and through the blood–brain barrier (Kang et al., 2000; Shibata et al., 2000; Van Uden et al., 2002), impaired γ-secretase function might affect A/β clearance. On the other hand, apo E also seems to alter the ratio of A/β40/A/β42, and promote aggregation and deposition of A/β (Bales et al., 2002; Holtzman, 2003; Dolev and Michaelson, 2006). Thus, the inefficient clearance of apo E could contribute to increased deposition of A/β in PS-dependent FAD. Together, our data provide a potential link between mutations in PS that cause early-onset AD and apo E that is critically involved in late-onset AD (Tanzi and Bertram, 2005). It is therefore intriguing to speculate that apo E-dependent lipid metabolism and A/β clearance in the brain contribute to the pathogenesis of early-onset FAD caused by PS mutations.

A recent study indicated that AICD could suppress LRP1 transcription (Liu et al., 2007). In addition, the increased LR1 expression in APP KO cells and embryonic mouse brain was associated with enhanced metabolism of apo E and decreased apo E levels (Liu et al., 2007). Our data do not support a role of AICD in the transcriptional regulation of LR1. No significant changes in LR1 mRNA levels were observed in brains of embryonic APP/APLP1/APLP2 KO mice that lack ICDs of all members of the APP family. However, a slight increase in LR1 mRNA levels in brains of adult APP KO mice was detected. Because this increase was not seen in APPsα/ALPL2−/− (DKI) mice, which lack the ICDs of APP and APLP2 but express soluble APP, these data rather could suggest an involvement of the APP ectodomain in the regulation of LR1 mRNA expression. The decreased uptake of apo E and cholesterol in neurons with impaired γ-secretase activity could impair cholesterol-dependent processes, including dendrite differentiation and synaptogenesis (Mauch et al., 2001; Pfrieger, 2002).

PS proteins have been previously implicated in the subcellular trafficking of membrane proteins, including tyrosinase, nicas- trin, N-cadherin, and telencephalin (Annaert et al., 2001; Wang et al., 2006; Zhang et al., 2006; Parks and Curtis, 2007). Consistent with our data, the endocytosis of BSA and APP was also

Figure 7. Accumulation of APP CTFs causes mistargeting of adaptor proteins and decreased endocytosis of LDLR. **A**, HEK293 cells were transfected with cDNA encoding myc-tagged Fe65. After 48 h, cells were fixed, permeabilized, and stained for Fe65 with anti-myc antibody 9E10. Cells were counterstained with DAPI to localize nuclei. **B**, Cells were transfected with cDNAs encoding APP C99-EGFP and myc-tagged Fe65, and then incubated in the presence (bottom) or absence (top) of 10 μM DAPT. Expression of C99-EGFP caused relocation of Fe65 to juxtanuclear compartments (top). In the presence of DAPT, prominent staining at the cell surface was also observed (bottom). **C, D**, APP CTF-dependent localization of ARH. HEK293 cells were transfected with cDNA encoding ARH alone (C) or together with APP C99-EGFP (D). Cells were incubated in the presence (D, bottom) and absence (D, top) of 10 μM DAPT. Scale bars in A–D, 15 μm. **E**, LDLR endocytosis in HEK293 control cells and in cells expressing APP C99 was analyzed by biotinylation as described under Materials and Methods. Quantitative ECL imaging revealed that C99 expression resulted in significantly decreased endocytosis of LDLR (34.3 ± 18.0% vs 100 ± 11.6%; p < 0.05).
found to be decreased in PS-deficient cells (Kaether et al., 2002; Wood et al., 2005), but the underlying molecular mechanisms were unclear. We show that accumulation of APP CTFs caused aberrant targeting of Fe65 and ARH to the plasma membrane. Thus, accumulated APP CTFs could engage a significant pool of these adaptor proteins, thereby compromising endocytosis of cell surface proteins.

Increased levels of APP CTFs have also been observed in cells expressing FAD-associated mutations in APP (McPhie et al., 1997). Because APP CTFs impair Ca2+ homeostasis, long-term potentiation, and inflammatory processes (Yankner et al., 1989; Berger-Sweeney et al., 1999; Lahiri et al., 2002; Chang and Suh, 2005), their accumulation could contribute to neuronal dysfunction in AD. Indeed, mice with PS deficiency in forebrain neurons show age-dependent accumulation of APP CTFs and neurodegeneration (Hermes et al., 2003; Saura et al., 2004), which because of the lack of γ-secretase could not be attributed to toxic effects of Aβ. Our data indicate that accumulation of these CTFs by (partial) loss of γ-secretase function also impairs lipoprotein uptake and cellular cholesterol homeostasis which might, in addition to altered Aβ production, contribute to the pathogenesis of AD. The number of known γ-secretase substrates is steadily increasing and includes cell adhesion molecules, surface receptors, and channel proteins (Wolfe and Kopan, 2004; Parks and Curtis, 2007). The ICDs released after γ-secretase cleavage of Notch, N- and E-cadherin, and ErbB4 could regulate gene transcription after translocation to the nucleus (Marmambaud et al., 2003; Koo and Kopan, 2004). Although AICD could affect gene transcription by association with Fe65 in vitro, its release from cellular membranes by γ-secretase appears to be not absolutely required (Biederer et al., 2002; Cao and Sudhof, 2004; Hass and Yankner, 2005). Thus, the γ-secretase-dependent cleavage of APP CTFs and probably other substrates might not be important for nuclear signaling, but rather serve for degradation of certain membrane proteins (Kopan and Ilagan, 2004; Sambamurti et al., 2006; Selkoe and Wolfe, 2007). Accumulation of other γ-secretase substrates might therefore also contribute to the pathogenesis of AD.

References


