Full-length amyloid precursor protein regulates lipoprotein metabolism and amyloid-β clearance in human astrocytes

Lauren K. Fong1,4, Max M. Yang1,4, Rodrigo dos Santos Chaves1,4, Sol M. Reyna1,4, Vanessa F. Langness1,4, Grace Woodruff1,4, Elizabeth A. Roberts1,4, Jessica E. Young1,3, and Lawrence S.B. Goldstein1,2,4*

From the 1Department of Cellular and Molecular Medicine, 2Department of Neurosciences, University of California, San Diego, 3Department of Pathology and Institute of Stem Cell and Regenerative Medicine, University of Washington, 4Sanford Consortium for Regenerative Medicine, La Jolla, CA 92093

Running Title: Astrocytic APP Controls LDL Receptor Function

*To whom correspondence should be addressed: Lawrence S.B. Goldstein: Department of Cellular and Molecular Medicine, Department of Neurosciences, University of California, San Diego, Sanford Consortium for Regenerative Medicine, La Jolla, California 92093; lgoldstein@ucsd.edu; Tel: (858) 534-9702; Fax: (858) 246-0162.

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ABSTRACT

Mounting evidence suggests that alterations in cholesterol homeostasis are involved in Alzheimer’s disease (AD) pathogenesis. Amyloid precursor protein (APP) or multiple fragments generated by proteolytic processing of APP have previously been implicated in the regulation of cholesterol metabolism. However, the physiological function of APP in regulating lipoprotein homeostasis in astrocytes, which are responsible for de novo cholesterol biosynthesis and regulation in the brain, remains unclear. To address this, here we used CRISPR/Cas9 genome editing to generate isogenic APP-knockout (KO) human induced pluripotent stem cells (hiPSCs) and differentiated them into human astrocytes. We found that APP-KO astrocytes have reduced cholesterol and elevated levels of sterol regulatory element-binding protein (SREBP) target gene transcripts and proteins, which were both downstream consequences of reduced lipoprotein endocytosis. To elucidate which APP fragments regulate cholesterol homeostasis and examine whether familial AD mutations in APP affect lipoprotein metabolism, we analyzed an isogenic allelic series harboring the APP Swedish and APP V717F variants. Only astrocytes homozygous for the APP Swedish (APPswe/swe) mutation, which had reduced full-length APP (FL APP) due to increased β-secretase cleavage, recapitulated the APP-KO phenotypes. Astrocytic internalization of amyloid-β (Aβ), another ligand for low-density lipoprotein (LDL) receptors, was also impaired in APP-KO and APPswe/swe astrocytes. Finally, impairing cleavage of FL APP through β-secretase inhibition in APPswe/swe astrocytes reversed the LDL and Aβ endocytosis defects. In conclusion, FL APP is involved in the endocytosis of LDL receptor ligands and required for proper cholesterol homeostasis and Aβ clearance in human astrocytes.

Alzheimer’s disease (AD) is a progressive and irreversible neurodegenerative disease that is the most common form of dementia in the elderly (1). Genetically, AD can be subdivided into two subgroups: early onset, familial Alzheimer’s disease (FAD) and late onset, sporadic Alzheimer’s disease (SAD) (2). While FAD can be attributed to rare and highly penetrant mutations in amyloid precursor protein (APP), presenilin-1, or presenilin-2, the precise etiology of SAD is unknown (3). Recently, several lines of evidence have implicated cholesterol metabolism as a common biological
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pathway involved in FAD and SAD. The ε4 allele of APOE, the major cholesterol carrier of the brain, is the strongest known genetic risk factor in AD (4, 5). Genome-wide association studies have identified additional mutations in lipid metabolism-related proteins like APOJ/Clusterin (CLU) and ABCA7 as other highly-associated risk factors (6). Finally, retrospective studies, although controversial, have suggested that use of the cholesterol-lowering statins lowers risk for AD (7–9).

APP is a ubiquitously expressed, single-pass, type I transmembrane protein, that is thought to have multiple putative biological functions (10). The functional heterogeneity of APP may stem from its multiple proteolytic fragments, which are generated by two major pathways: non-amyloidogenic cleavage via sequential proteolysis by α- and then γ-secretase, or amyloidogenic cleavage by β- and then γ-secretase. APP or various proteolytic fragments of APP, have recently been implicated in the control of brain cholesterol metabolism via regulation of LDL receptor-family mRNA and protein (11–15). Normally, low intracellular cholesterol levels induce cholesterol biosynthesis via intricate transcriptional and posttranslational mechanisms involving increased proteolytic processing of SREBPs, which result in upregulated cholesterol biosynthesis enzymes and increased internalization of extracellular cholesterol via LDL receptors. In contrast, high intracellular cholesterol levels inhibit the proteolytic processing of SREBP and thus turn off cholesterol biosynthesis and uptake (16). Regulation of LDL receptor mRNA and protein is also relevant to mechanisms of Aβ clearance in the brain since LDL receptors like low-density lipoprotein receptor (LDLR) and lipoprotein receptor-related protein 1 (LRP1) mediate the internalization of Aβ by binding to Aβ directly or via apoE (17–21).

Despite our understanding of the sensitive and complex mechanisms regulating intracellular cholesterol, there is little consensus as to how APP regulates this pathway in the brain. Multiple studies on these subjects have yielded mixed results, likely due to the use of non-endogenous APP levels, the study of non-neural cell types, or the use of whole brain tissue, which masks the unique phenotypes of individual cell types. To begin to address these issues, we utilized CRISPR/Cas9 genome editing to generate an isogenic series of APP-KO and FAD mutant hiPSCs. We further differentiated these hiPSCs into astrocytes, the cell type primarily responsible for the brain’s de novo cholesterol synthesis and regulation. Using APP-KO, APP Swedish, and APP V717F astrocytes, we identify a role for FL APP in the uptake of LDL receptor ligands and demonstrate that proper levels of FL APP in human astrocytes are essential for lipoprotein regulation and Aβ clearance. Our data shed light onto the elusive function of FL APP, establish a linkage between APP and biological pathways implicated in SAD, and lastly, highlight the utility of using hiPSC technology to study the physiological function of endogenous proteins in specific cell types.

RESULTS

Generation of isogenic APP knockout hiPSCs using CRISPR/Cas9—To study the role of endogenous human amyloid precursor protein (APP) in regulating astrocytic cholesterol metabolism, we utilized CRISPR/Cas9 genome editing to knockout (KO) APP in human induced pluripotent stem cells (hiPSC) (Figure 1). To induce gene disruption, we utilized a guide RNA targeting exon 16 of APP. In both APP-KO clones used in our experiments, the CRISPR/Cas9-guide RNA complexes generated unique indels at the predicted cut site in each allele. All of these indels were predicted to generate premature stop codons in either exon 16 or exon 17 of APP (Figure 2A). For analysis, we compared these APP-KO hiPSCs to WT, unedited subclones of the original hiPSC line that also underwent the genome editing process, but were not modified.

To test whether pre-mature stop codon formation had induced nonsense-mediated decay (NMD) of APP mRNA and thus loss of APP protein, we first examined APP transcript using primer sets targeting regions upstream and downstream of the CRISPR/Cas9 cut site. There was little detectable APP mRNA in APP-KO neural precursor cells (NPCs), neurons, and astrocytes (Figure 2B-2D). Consistent with these results, two N-terminal full-length APP (FL APP) antibodies, which recognized epitopes either upstream (22C11) or downstream (6E10) of the
CRISPR/Cas9 cut site, and one C-terminal APP antibody (APP CTF) failed to detect any APP protein in APP-KO NPCs (Figures 2E and S1). Furthermore, the medium from APP-KO neurons contained no detectable cleaved APP fragments like Aβ or the secreted soluble APP (sAPP) fragments (data not shown). Together these data suggested that CRISPR/Cas9-induced NMD of APP transcripts prevented translation of APP protein in APP-KO NPCs, neurons, and astrocytes.

We also examined the expression of APP family members, Amyloid Precursor-Like Protein 1 and 2 (APLP1 and APLP2), which have been shown to exert some functional redundancy with APP. Interestingly in APP-KO NPCs, we saw a modest upregulation of APLP1 and APLP2 mRNA levels (Figure 2B) and protein (Figure 2E). However, when these APP-KO NPCs were further differentiated to neurons (Figure 2C) or astrocytes (Figure 2D), this upregulation of APLP1 and APLP2 mRNA was no longer detected. These data indicated that there were no off-target effects on homologous APP family members and any phenotypes observed in APP-KO neurons or astrocytes were not due to loss or overexpression of APLP1 or APLP2.

Because APP is a key protein in neural development (22), we next tested whether the loss of APP influenced the capability of APP-KO NPCs to differentiate into neurons or astrocytes. Using flow cytometry for a neuronal cell surface signature of CD184+, CD44+, and CD24+ (23), we found no difference in the percentage of neurons generated from WT or APP-KO NPCs following multiple rounds of neuronal differentiation (Figure 2F). Similarly, using the glial markers, CD44, CD184, and GFAP, we found no difference in the differentiation capability of WT or APP-KO NPCs to generate astrocytes (Figure 2G).

hiPSC-derived astrocytes produce high levels of de novo synthesized cholesterol in vitro—Given the relative isolation of the brain from the periphery because of the blood brain barrier, cholesterol is synthesized locally in the brain (24). Previous studies examining sterol synthesis in brain-specific cell types have identified astrocytes as the brain’s primary source of cholesterol (25). To test whether our hiPSC-derived system recapitulated this essential function of astrocytes in vivo, we performed a comprehensive analysis of free sterols in astrocytes, neurons, and NPCs using liquid chromatography/mass spectrometry. We found that, relative to neurons and NPCs, astrocytes had significantly elevated levels of the immediate cholesterol precursors, desmosterol (Figure 3A) and 7-dehydrocholesterol (Figure 3B). Consistent with previous work reporting that astrocytes predominantly contain sterols from the Bloch pathway of cholesterol biosynthesis (26), we observed higher concentrations of astrocyte-derived desmosterol relative to 7-dehydrocholesterol. These data suggest that hiPSC-derived astrocytes recapitulate the ability to synthesize cholesterol, similar to their native function in vivo.

APP-KO astrocytes have altered cholesterol metabolism—We next sought to determine if loss of APP affected cholesterol levels in hiPSC-derived astrocytes. Compared to WT, APP-KO astrocytes had decreased cholesterol (Figure 3C). Given that levels of intracellular cholesterol regulate the activity of the sterol regulatory element-binding protein (SREBP) family of transcription factors (27, 28), we next analyzed SREBP-target genes. These included HMG-CoA reductase (HMGR), the rate-limiting enzyme in cholesterol biosynthesis, and the low-density lipoprotein receptor (LDLR). We also analyzed expression of low-density lipoprotein receptor-related protein 1 (LRP1), another highly expressed lipoprotein receptor in human astrocytes (29), whose expression has been reported to be regulated by the APP intracellular domain (AICD) (12) in addition to SREBP (30). mRNA expression of HMGCR, LDLR, and LRP1 was upregulated in APP-KO astrocytes when grown in normal growth medium with 3% serum (Figure 3D). To test whether this result in APP-KO astrocytes was a consequence of low intracellular cholesterol and not simply aberrant SREBP function, we examined whether APP-KO astrocytes could further upregulate transcript in response to prolonged lipoprotein depletion. Indeed, after culture under lipoprotein-free conditions, differences in SREBP-target gene expression were no longer observed between WT and APP-KO astrocytes (Figure 3E). Furthermore, there was no difference between WT and APP-KO astrocytes in the fold
induction of either HMGCR, LDLR, or LRP1 mRNA after 24 hours of cholesterol withdrawal (Figure 3F). These data suggested that since APP-KO astrocytes could still modulate SREBP-target gene expression, low intracellular cholesterol resulted in upregulation of cholesterol synthesis and internalization genes.

**APP-KO astrocytes have decreased lipoprotein endocytosis, but are not defective in bulk endocytosis, bulk receptor recycling, or expression of LDL receptors at the cell surface**—Because APP-KO astrocytes exhibited a cholesterol starvation phenotype, but did not demonstrate impaired SREBP signaling in response to cholesterol withdrawal, we hypothesized that APP-KO astrocytes had reduced endocytosis of extracellular lipoproteins from the culture media. To test this, WT and APP-KO cells were treated with fluorescently-labeled LDL (Figure 4A). After one hour of continuous LDL treatment, APP-KO astrocytes demonstrated a modest, but significant reduction in lipoprotein endocytosis as reflected by a reduction in intensity of intracellular LDL fluorescence. To test whether this reduction in lipoprotein endocytosis was a result of reduced bulk endocytosis, we treated cells with fluorescently-labeled dextran (Figure 4B). We found no difference in dextran internalization between WT and APP-KO astrocytes, suggesting that impaired endocytosis was specific for lipoproteins.

Next, to understand the mechanism of reduced lipoprotein endocytosis in APP-KO astrocytes, we also tested bulk receptor recycling by flow cytometry using fluorescently-labeled transferrin (Tfn). Tfn marks recycled cargo and allows for characterization of recycling compartments (31) and APP is known to recycle back to the cell surface in Tfn receptor-positive vesicles. To test the endocytic recycling of receptors at the cell surface, astrocytes were incubated with Tfn at 37°C for 10 minutes to allow Tfn uptake. Cells were then acid-washed to remove surface-bound Tfn before being chased with growth medium at fixed time points to allow Tfn to be recycled back to the cell surface. We observed no difference in the rate of Tfn recycling between WT and APP-KO astrocytes over time (Figure 4C), suggesting that general recycling of receptors in endosomes which normally contain APP was not impaired in APP-KO astrocytes.

Given that both bulk endocytosis and Tfn receptor-marked recycling pathways were not defective in APP-KO astrocytes, we next examined whether newly synthesized lipoprotein receptors could be shuttled to the cell surface via the secretory pathway. To do this, we measured cell surface LDLR by flow cytometry after treatment with berberine (BBR), which stimulates LDLR mRNA expression and upregulates cell surface LDLR (32). Though BBR treatment upregulated surface LDLR levels compared to DMSO treated astrocytes (Figure 4D), there were no differences in cell surface LDLR between WT or APP-KO astrocytes in either condition. To further verify that the loss of APP does not affect cell surface LDL receptor levels, we used a cell surface biotinylation assay to label plasma membrane bound proteins with a cleavable biotin and then used streptavidin beads to pull down surface proteins. This allowed us to measure cell surface (pull-down fraction), total (input fraction), and intracellular (supernatant fraction) LDLR and LRP1 levels via Western Blot (Figures 4E-4P). Confirming our previous results, we saw no difference in the amount of cell surface LDLR or LRP1 protein (Figures 4E-4H). However, in line with upregulated LDLR and LRP1 transcript in APP-KO astrocytes, we observed increased total LDLR and LRP1 protein (Figures 4I – 4L). Consistent with these results, intracellular LDLR and LRP1 protein was also elevated (Figures 4M - 4P). Finally, examination of APP-KO astrocytes by immunofluorescence revealed the presence of enlarged LRP1 puncta (Figure 4Q).

Together, these data revealed no defects in regulation of transcription, Tfn-receptor recycling pathways, or the shuttling of newly synthesized receptors to the cell surface via the secretory pathway in APP-KO astrocytes. These data suggest that the loss of APP attenuated lipoprotein endocytosis and contributed to decreased cholesterol and increases in SREBP-target genes.

**FAD astrocytes exhibit alterations in APP processing**—In light of previous work implicating multiple APP fragments in the regulation of cholesterol homeostasis, we aimed to determine which APP fragments are required
for proper lipoprotein metabolism. We also sought to examine whether FAD mutations in APP affect cholesterol homeostasis in human astrocytes. To do this, we analyzed an isogenic allelic series of astrocytes either heterozygous or homozygous for the APP Swedish mutation (APP\textsuperscript{Swe/WT}, APP\textsuperscript{Swe/Swe}) or APP V717F mutation (APP\textsuperscript{V717F/WT}, APP\textsuperscript{V717F/V717F}) (33). The Swedish and V717F mutations are thought to have different defects in APP processing which would allow us to make distinct predictions about which APP fragments are relevant to lipoprotein regulation (34, 35).

To characterize the APP processing alterations in FAD mutant astrocytes, we quantified protein levels of FL APP (Figure 5A). Of all FAD genotypes, only APP\textsuperscript{Swe/Swe} astrocytes exhibited a reduction of FL APP (Figure 5B). However, given that β-secretase processing of FL APP is favored in the APP Swedish mutation (36, 37), we hypothesized that this loss of FL APP in APP\textsuperscript{Swe/Swe} astrocytes would coincide with increases in Aβ and soluble APP (sAPP) β along with a decrease in sAPPα. As predicted, APP Swedish astrocytes secreted high levels of Aβ40, Aβ42, and Aβ38 (Figures 5F-5H), with no change in the Aβ42:Aβ40 ratio compared to WT (Figures 5E). Additionally, APP Swedish astrocytes exhibited decreases in sAPPα (Figure 5I), little detectable WT sAPPβ (Figure 5J), and increased Swedish sAPPβ (Figure 5K), which was recognized by an antibody specific for the APP Swedish mutation and only detectable in APP Swedish astrocytes. To detect APP C-terminal fragments (APP CTFs), astrocytes were treated with a γ-secretase inhibitor for 48 hours. Only APP Swedish astrocytes exhibited increased APP β-CTF fragments (Figure 5D) and reductions in the APP α-CTF fragment (Figure 5C). In APP V717F astrocytes, by contrast, we observed a dose-dependent increase in the Aβ42:Aβ40 ratio (Figure 5E) as a result of increased Aβ42 (Figure 5G) and no significant change in the amount of APP CTFs compared to WT after 48 hours of γ-secretase inhibitor treatment (Figure 5C and 5D).

APP V717F astrocytes also showed no changes in the levels of the soluble fragments, sAPPα (Figure 5I) or sAPPβ (Figure 5J). Collectively, these data are consistent with previous data reporting that the APP Swedish mutation enhances β-secretase cleavage of APP (36, 37) and the APP V717F mutation promotes an increased Aβ42:Aβ40 ratio (38, 39). We hypothesized that these distinct alterations in APP processing could help us elucidate which APP fragment is most important in regulating lipoprotein metabolism in FAD astrocytes.

**APP\textsuperscript{Swe/Swe} astrocytes recapitulate APP-KO phenotypes of impaired lipoprotein endocytosis and altered cholesterol metabolism**—To examine cholesterol homeostasis in APP mutant FAD astrocytes, we tested lipoprotein endocytosis in which we had previously observed a defect in APP-KO astrocytes. Of all APP mutant genotypes, only APP\textsuperscript{Swe/Swe} astrocytes phenocopied APP-KO astrocytes in reduced lipoprotein endocytosis (Figure 6A) without a concomitant reduction in bulk endocytosis (Figure 6B). APP-KO, APP\textsuperscript{Swe/WT}, and APP\textsuperscript{Swe/Swe} astrocytes all exhibited reductions in the APP fragments generated by α-secretase cleavage (Figure 5C and 5I). However, given that only APP\textsuperscript{Swe/Swe}, but not APP\textsuperscript{Swe/WT} astrocytes mimicked APP-KO phenotypes, we hypothesized that FL APP might be crucial in regulating lipoprotein metabolism.

To determine if reduced lipoprotein endocytosis in FAD astrocytes with reduced FL APP levels also led to downstream alterations in cholesterol metabolism, we looked at the expression of multiple proteins involved in lipoprotein regulation (Figure 6C). We first examined the transcription factor SREBP1, which regulates intracellular cholesterol levels. SREBP function is controlled by multiple mechanisms including self-regulation by transcriptional positive feedback, and activation via sequential proteolysis and translocation of its mature, cleaved fragment to the nucleus. Protein levels of both full-length (FL) (Figure 6D) and cleaved SREBP1 (Figure 6E) were elevated in APP-KO and APP\textsuperscript{Swe/Swe} astrocytes. In response to attenuated lipoprotein endocytosis, we observed that the ratio of cleaved/FL-SREBP protein was also significantly increased in APP-KO and APP\textsuperscript{Swe/Swe} astrocytes (Figure 6F). Since mature SREBP also upregulates lipoprotein receptor-mediated uptake of extracellular lipoproteins, we further examined LDLR protein in FAD astrocytes. As previously observed in APP-KO astrocytes, LDLR protein was elevated in APP\textsuperscript{Swe/Swe} astrocytes (Figure 6G). Together,
these data suggest that both APP-KO and APP\textsuperscript{Swe/Swe} astrocytes have impaired lipoprotein endocytosis and exhibit downstream biochemical changes expected in cholesterol-deficient cells.

**APP-KO astrocytes and APP\textsuperscript{Swe/Swe} astrocytes also have impaired uptake of Aβ, another LDL-receptor ligand**—Given that reduction of FL APP coincided with impaired lipoprotein endocytosis and cholesterol homeostasis, we speculated that FL APP might also be required for other astrocyte-specific functions related to lipoprotein receptor function. We hypothesized that astrocytes with reduced FL APP would also be defective in lipoprotein receptor-mediated internalization of the Aβ peptide.

To determine whether our hiPSC-derived astrocytes could internalize Aβ, WT astrocytes were treated with FITC-conjugated Aβ for 15 minutes, washed, and given fresh medium (Figure 7A). Over the course of 72 hours, we examined the presence of Aβ-FITC, early endosome marker EEA1, and M6PR which tags vesicles destined for transport to the lysosome. While the amount of EEA1 and M6PR puncta remained constant, the number of Aβ-FITC puncta decreased over time (Figure 7B). Further analysis demonstrated that Aβ colocalization with M6PR increased over time (Figure 7C), suggesting that Aβ was being targeted for lysosomal degradation. We further verified this observation using flow cytometry of WT astrocytes treated with the pH-sensitive Aβ-FITC or the pH-insensitive Aβ-HiLyte Fluor 647 (Figure 7D). Both probes were utilized since a pH-sensitive signal will decrease in fluorescence intensity when it reaches a more acidic compartment like the lysosome (40). Over the course of 48 hours, the intensity of both Aβ-FITC and Aβ-HiLyte Fluor 647 increased over time. However, at 72 hours we observed a reduction in the pH-sensitive Aβ-FITC, but not Aβ-HiLyte Fluor 647, suggesting that Aβ was being targeted to an acidic compartment following internalization. To exclude the possibility that the reduction of Aβ-FITC simply reflected an inability to detect the probe, but not actual Aβ degradation, we supplemented astrocyte culture medium with Aβ for 24 hours and measured the concentration of Aβ in astrocytes over time (Figure 7E). Over the course of 48 hours, we observed a 90% reduction in Aβ. Together these data indicate that WT hiPSC-astrocytes could both internalize and degrade Aβ. To test whether APP-KO and APP\textsuperscript{Swe/Swe} astrocytes are defective in Aβ internalization in addition to lipoprotein endocytosis, we treated astrocytes with Aβ-HiLyte Fluor 647 for 24 hours of continuous uptake (Figure 7F). We observed reduced internalization of Aβ in both APP-KO and APP\textsuperscript{Swe/Swe} astrocytes, but not in APP V717F astrocytes, indicating that normal levels of FL APP are required for proper LDL receptor function in the endocytosis of both extracellular lipoproteins and Aβ.

β-Secretase inhibitor treatment reverses impairments in lipoprotein and Aβ endocytosis in APP\textsuperscript{Swe/Swe} astrocytes—Because APP\textsuperscript{Swe/Swe} astrocytes recapitulated defects observed in APP-KO cells, we hypothesized that this was a consequence of increased cleavage and loss of FL APP protein by β-secretase. Since FL APP is transported away from the plasma membrane in an endocytic compartment for β-secretase cleavage (41, 42), we predicted that APP\textsuperscript{Swe/Swe} astrocytes also had reduced APP at the cell surface. Using cell surface biotinylation and streptavidin beads to pull down surface proteins (Figure 8A), we find that approximately 10% of total cellular APP is present at the cell surface in WT astrocytes (Figure 8B). However, in APP\textsuperscript{Swe/Swe} astrocytes, we observed a 50% reduction of cell surface APP compared to WT.

To test our hypothesis that normal levels of FL APP are required for proper lipoprotein metabolism and Aβ clearance, we utilized a β-secretase inhibitor (BSI) to inhibit β-cleavage in APP\textsuperscript{Swe/Swe} astrocytes. Because BSIs are sometimes reported to have low potency in cells expressing the APP Swedish mutation (43), we measured whether 24 hours of BSI-treatment could reduce Aβ secretion in WT and APP\textsuperscript{Swe/Swe} astrocytes. We observed decreased Aβ40 peptides in BSI-treated WT astrocytes and a marked reduction of Aβ40 in BSI-treated APP\textsuperscript{Swe/Swe} astrocytes near WT levels (Figure 8C). To test whether BSI-treatment could rescue the deficits we observed in APP\textsuperscript{Swe/Swe} astrocytes, we treated WT, APP-KO, and APP\textsuperscript{Swe/Swe} astrocytes with a BSI for 24 hours and measured LDL and Aβ endocytosis. Upon pharmacological inhibition of β-secretase in APP\textsuperscript{Swe/Swe} astrocytes,
we observed a reversal of defects in both LDL endocytosis (Figure 8D) and Aβ endocytosis (Figure 8E). Significant increases in LDL or Aβ endocytosis were not observed in the absence of APP, indicating that rescue of impaired endocytosis relied on impairing β-secretase cleavage of FL APP.

DISCUSSION

APP is a transmembrane protein that is highly expressed in the central nervous system. It has been shown to have many varied biological functions, likely due to multilayered mechanisms of regulation resulting in multiple proteolytic products and alternatively spliced isoforms. To determine whether APP or any of its proteolytic cleavage products are involved in lipoprotein regulation, we employed CRISPR/Cas9-genome editing to generate APP-KO, APP Swedish, and APP V717F hiPSCs and differentiated them into human astrocytes, the source of de novo cholesterol in the brain. Here we show that FL APP regulates LDL receptor function. Loss of FL APP resulted in impaired lipoprotein and Aβ endocytosis, reduced intracellular cholesterol, and aberrant elevations of transcripts and proteins related to cholesterol synthesis and internalization. Finally, we show that inhibiting cleavage of FL APP by β-secretase can reverse LDL and Aβ endocytosis defects, but only in the presence of APP. Thus, in addition to having a critical role in mammalian brain development (44), normal levels of FL APP are also critical in the maintenance of homeostatic brain functions. We propose that pathological alterations of FL APP levels could contribute to glial dysfunction in multiple neurodegenerative disorders.

Mechanistically, we attribute defective lipoprotein and Aβ endocytosis in APP-KO and APP\textsuperscript{Swe/Swe} astrocytes to the loss of FL APP in each of these genotypes. APP isoforms containing the KPI-domain have been shown to bind to LR1 at the N-terminus (45, 46). Intracellularly, the cytoplasmic adaptor protein FE65 has been shown to link the C-terminal NPXY endocytosis motifs of APP and multiple LDL receptors (47–49). Thus, astrocytic APP isoforms, which include the KPI-domain, may have a dual linkage with LDL receptors at both the N- and C-termini. In light of this prior work demonstrating interactions between APP and LDL receptors, it is possible that FL APP acts as a co-receptor for LDL receptor ligands in human astrocytes. A further explanation for dysregulated cholesterol metabolism in APP-KO and Swedish astrocytes is that these genotypes also have a high rate of ligand-independent receptor endocytosis. It is feasible that both decreased endocytosis of lipoproteins and increased ligand-independent endocytosis of the LDL receptors combine in an additive manner to produce the results we observed. Overall, it is consistent with our data that without proper FL APP levels, that the function of LDL receptors is impaired. This dovetails nicely with previous studies demonstrating that APP interacts with diverse binding partners including: APOE (50), kinesin (51, 52), SORLA (53), and cholesterol (54). Interestingly, all of these interacting partners modulate APP metabolism and alterations in these interactions are hypothesized to contribute to AD pathology.

**FL APP regulates brain cholesterol metabolism**—In human astrocytes, we find that normal levels of FL APP are essential for proper regulation of cholesterol homeostasis. Loss of FL APP led to impairments in lipoprotein endocytosis (Figure 4A), resulting in decreased intracellular cholesterol (Figure 3C) and activation of SREBP-target gene transcripts and protein (Figures 3D, 4I – 4L) in both APP-KO and Swedish astrocytes. Interestingly, we did not observe a dose-dependent effect of FL-APP levels in our LDL endocytosis experiments as we did in the Aβ endocytosis experiments. While both APP-KO and Swedish astrocytes exhibited differential handling of lipoproteins compared to WT astrocytes, it is possible that more subtle changes in lipoprotein endocytosis correlating with FL APP levels are below the level of detection in the assay.

Previous work done by our lab using the same isogenic FAD hiPSC lines to study human neurons revealed that accumulation of a different APP fragment, β-CTF, caused impairments in lipoprotein endocytosis and a neuron-specific transcytotic trafficking pathway via defects in recycling (33). Although we cannot rule out that the overabundance of APP β-CTF in APP\textsuperscript{Swe/Swe} astrocytes outcompetes FL APP for binding to LDL receptors and thus impairs recycling of LDL receptors to the surface, CTFs were difficult to
detect in our hiPSC-derived astrocytes without γ-secretase inhibition. This suggests that steady-state levels of the rapidly processed or degraded β-CTF might be quite low relative to FL APP or Aβ in APP<sup>Swe/Swe</sup> astrocytes. Given the low levels of astrocytic β-CTF we observed, we hypothesize that we might not observe cholesterol phenotypes in FAD astrocytes which are characterized by accumulation of β-CTF protein, like presenilin mutant astrocytes. By contrast, in mutant astrocytes characterized by increased APP expression (e.g., APP duplication or Down Syndrome), we hypothesize that there will be alterations in cholesterol metabolism. Altering APP gene dosage in astrocytes is likely to alter its normal physiologic processing as well as the processing of other proteins, like LRP1, with which APP may be competitive substrate. Collectively, these data shed light on the importance of studying the role of endogenous proteins in specific cell types as we find that alterations in APP processing can affect either astrocyte or neuron-specific functions via different mechanisms. Increased β-CTF in human neurons and loss of FL APP in human astrocytes may impair lipoprotein endocytosis independently in a cell-specific context. Additional work comparing astrocytes and neurons from different FAD mutations would be revealing.

In this study we demonstrate a linkage between FL APP levels and lipoprotein endocytosis. However, further study of how FL APP levels in astrocytes influence lipoprotein export and thus neuronal health is also needed, given the reliance of neurons on astrocyte-derived lipoproteins (25, 56). Future work to investigate how aberrant lipoprotein metabolism in astrocytes contributes to AD phenotypes could provide mechanistic insight into the development of new AD therapeutics.

**FL APP regulates Aβ clearance**—Here we report that loss of FL APP in APP-KO and APP<sup>Swe/Swe</sup> astrocytes impairs Aβ internalization (Figure 7F). Interestingly, we do not observe Aβ clearance defects in the other FAD mutations. This not only reflects the high degree of clinical and pathological heterogeneity within AD, but also heterogeneity within subgroups of FAD patients harboring different FAD mutations (57, 58). Though it is possible that some FAD mutant astrocytes could have small alterations in FL APP levels that are also below our level of detection, these phenotypic differences are also in agreement with notion that the accumulation of Aβ in FAD is primarily due to neuronal overproduction of Aβ. In line with this idea, FAD mutations in APP are often considered to be gain of function mutations due to the generation and accumulation of some toxic proteolytic product (3). However, here we find in APP<sup>Swe/Swe</sup> astrocytes that the mutation confers a loss of function phenotype associated with the loss of FL APP to β-secretase cleavage. We postulate that the impairment in Aβ clearance in APP<sup>Swe/Swe</sup> astrocytes could also contribute to the greatly increased Aβ plaque load observed in mouse models overexpressing the APP Swedish mutation (59–61).

In light of data suggesting that the increased deposition of Aβ in SAD is primarily a result of impaired Aβ clearance rather than increased Aβ generation (62), further work in this system could examine whether astrocytes derived from SAD patients are defective in Aβ clearance. Also, given the observation that both reactive astrocytes and cells undergoing a cellular stress response alter APP expression and APP processing (63–65), it would be revealing to study how FL APP levels under these pathological conditions affect Aβ clearance mechanisms.

**Linking APP to mechanisms of glial dysfunction in SAD**—In this study, we find that loss of FL APP impairs LDL receptor function. We provide a novel linkage between FL APP levels and two potential mechanisms of glial dysfunction in AD: dysregulation of cholesterol metabolism and Aβ clearance. While several studies have addressed the effect of the loss of LDL receptors on APP processing (66–69), little has been done to elucidate how the loss of APP affects LDL receptor function. The concept that FL APP levels directly affect the ability of LDL receptors to endocytose lipoproteins or Aβ is in agreement with the known stoichiometry of the LRP1-FE65-APP trimeric complex (47). Loss or overproduction of any member of this multimeric complex could alter the various functions of the LDL receptors by abrogating complex formation. Together, using endogenous protein levels in an isogenic series of iPSC-derived human...
astrocytes, our data shed light on the novel function of FL APP in controlling LDL receptor-mediated cholesterol metabolism and Aβ clearance in human astrocytes. These findings suggest that FL APP may have a more central role in the etiology of AD than previously suspected.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—iPSCs were cultured as previously described (70–72) on a MEF feeder layer in HUES medium: Knockout DMEM, 10% knockout serum replacement, 10% plasmanate (Grifols Therapeutics, Inc.), 1x non-essential amino acids, 1x glutamax, 1x Pen/Strep, and 0.1 mM β-mercaptoethanol (all Invitrogen) with 20 ng/ml FGF-2 (Millipore). iPSCs were passaged with Accutase (Innovative Cell Technologies) and supplemented with 10 µM Rock Inhibitor (RI, Y-27632 dihydrochloride, Abcam) after passaging only. iPSCs were differentiated into NPCs as previously described (23) by seeding iPSCs on a PA6 stromal cell layer in PA6 differentiation medium: Glasgow DMEM, 10% knockout serum replacement, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 0.1 mM β-mercaptoethanol (all Invitrogen), in the presence of 500 ng/ml Noggin (R&D Systems) and 10 mM SB431542 (Stemgent) for 6 days. Following 12 to 14 days of differentiation, NPCs were FACS purified using a CD184-APC+, CD271-PE−, CD44-PE−, CD24-PECy7+ (BD Biosciences) cell surface signature on a BD FACSAria II flow cytometer. NPCs were cultured on 20 mg/ml poly-L-ornithine/laminin-coated 10 cm plate. One week later, after which astrocytes emerged from the neurospheres, the cells were passaged with Accutase, cultured in AGM, and maintained with the neurospheres. To make lipoprotein free astrocyte medium, FBS was replaced with Lipoprotein Deficient Serum (Sigma). For experimental use, neurospheres were not included. In every experiment, each genotype is represented by the following number of independently differentiated hiPSC subclones/astrocyte lines: WT (2 or 3 clones: 151, IB7, IID4), APP-KO (2 clones: IA1, IB6), APP^Swe^WT (2 clones: 12-2, 12-7), APP^Swe^Swe (2 clones: 22, 119), APP^V717F^WT (1 clone: IVD8), APP^V717F/V717F (1 clone: IIIB12).

**Genome Editing**—All isogenic iPSCs were derived using CRISPR/Cas9 as previously described from the CVB iPSC line (33, 71) derived from J. Craig Venter (73), whose diploid genome sequence is publicly available (74). In brief, iPSCs were pretreated with 10 µM RI prior to nucleofection. To obtain single cells, iPSCs were dissociated with Accutase and filtered twice through 100 µM filters. To generate APP-KO and APP V717F clones, 2 × 10^6 iPSC were nucleofected using the Amaxa Human Stem Cell Nucleofector Kit I (Lonza) with 6 µg CMV::Cas9-2A-eGFP vector and 3 µg U6::gRNA vector, which was generated using the gRNA synthesis protocol as previously described (75). For APP Swedish clones, 8 × 10^5 iPSC were nucleofected with 5 µg pSpCas9(9BB)-2A-GFP (PX458) vector, which was generated as previously described (76). The following guide RNA sequences were used: targeting exon 16 for APP-KO and APP Swedish: GGA GAT CTC...
TGA AGT GAA GAT GG; and exon 17 for APP V717F: GAC AGT CAT CAC CTT GG. To introduce the APP Swedish or APP V717F point mutations in our WT CVB hiPSC line using CRISPR/Cas9-directed repair, 100 μM of single-stranded DNA oligonucleotides was also included during nucleofection. After culturing the iPSCs in the presence of RI for 48 to 72 hours, 1 × 10⁶ GFP+ iPSCs were FACS sorted (FACS Aria Ilu, BD Biosciences) and plated on 10 cm MEF feeder plates in the presence of RI. After one week, single colonies were manually picked, cultured in 96-well plates, and expanded. DNA from single clones was harvested using QuickExtract DNA Extraction Solution (Epicentre) and PCR amplified using the following PCR primers: APPex16-F: CCC GTA AGC CAA GCC AAC AT, APPex16-R: CAT CGA CGA ACT TTG CTG CC, or APPex17-F: TGT TCC ACC TGT CAA AGG GT, APPex17-R: AGT TGA GAT ACA GG. PCR products were purified using the QiAquick PCR Purification Kit (Qiagen) or ExoSAP IT PCR Product Cleanup Reagent (Thermo Fisher) as directed by the manufacturer and Sanger sequenced. Clones in which disruptions at the predicted gRNA/Cas9 cut site were observed were further sequenced after cloning using the Zero BluTOPO PCR Cloning Kit (Invitrogen). All previously unpublished isogenic iPSCs were digitally karyotyped by hybridization to the Infinium CoreExome-24 BeadChip (Illumina) as described previously (77) and determined to be euploid.

**RNA Expression**—For mRNA expression analysis, RNA was isolated using the RNeasy Mini Kit (Qiagen) and DNase-treated using TURBO DNase (Ambion) for one hour at 37°C. cDNA was synthesized from RNA primed with oligo(dT) using the SuperScript First-Strand Synthesis System (Invitrogen). QRT-PCR was performed using FastStart SYBR Green (Roche) and samples were run in triplicate on an Applied Biosystems 7300 RT-PCR system. Data was analyzed using the ΔΔCt method and target genes were normalized to the geometric mean of three housekeeping genes: RPL13A, RPL27, and TBP.

The following primers were used:

3′ **APP-F**: ATC ATG GTG TGG TGG AGG TT, 3′ **APP-R**: ACA CCG ATG GGT AGT GAA GC, 5′ **APP-F**: GAA GCA GCC AAT GAG AGA CAG, 5′ **APP-R**: TCA AAA TGC TTT AGG GTG TGC, **APLP1-F**: CTT CCC ACA GCC AGT AGA TGA, **APLP1-R**: CCA GGC ATG CCA AAG TAA ATA, **APLP2-F**: CCA TGG CAC TGA ATA TGT GTG, **APLP2-R**: CCT CAT CCT CAT CCA CAG, **HMGCR-F**: TCC CGT GGA AGT CAT AGT GG, **HMGCR-R**: AGG ATG GCT ATG CAT GTG, **LDLR-F**: CTG GAA ATT GCG CTG GAC, **LDLR-R**: GTC TTG GCA CTG GAA CTC GT, **LRP1-F**: CCA GCC CTG TGA TAT GAC AGG, **LRP1-R**: CTG CTC TCA GCT CTG GCC, **RPL13A-F**: GGA CCT CTG TGT ATT TGT CAA, **RPL13A-R**: GCT GGA AGT ACC AGG CAG TG, **RPL27-F**: AAA CCG CAG TTT CTG GAA GA, **RPL27-R**: TGG ATA TCC CCT TGG ACA AA, **SREBF2-F**: GAG ACC ATG GAG ACC CTC AC, **SREBF2-R**: TCA GGG AAC TCT CCC ACT TG, **TBPM-F**: TGC TTC ATA AAT TTC TG TCT G, **TBPM-R**: TAG AAG GCC TTG TG CAA CC.

**Protein Expression**—Cells were lysed in RIPA Lysis Buffer (Millipore) with protease (Calbiochem) and phosphatase inhibitors (Halt). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher). Equal amounts of protein lysates were run on NuPAGE 4-12% Bis-Tris gels (Invitrogen), transferred to nitrocellulose or PVDF membranes, and blocked for one hour at room temperature using either 5% milk or Odyssey Blocking Buffer (LI-COR). Blots were probed overnight at 4°C using the corresponding primary antibodies followed by HRP-conjugated (Vector Labs) or IRDye secondary antibodies (LI-COR) at 1:5,000. Bands were quantified using ImageJ software or the Odyssey Imaging System following manufacturer’s instructions. The following antibodies were used: anti-Actin (1:50,000; EMD Millipore MAB1501), anti-APLP1 (1:1,000; Calbiochem 171615), anti-APLP2 (1:1,000; Calbiochem 171616/7), anti-APP A4 clone 22C11 (1:1,000; EMD Millipore MAB348), anti-APP C-Terminal (1:500; EMD Millipore 171610), anti-β-Amyloid, 6E10 (1:1,000, BioLegend 803015), anti-LDLR (1:1,000; Abcam AB14056), anti-LRP1 (1:1,000; Abcam AB92544), and anti-SREBP1 (1:500; Proteintech 14088-1-AP).

**Sterol Analysis**—For free sterol analysis, 1 × 10⁶ cells were pelleted and stored at -80°C before sterols were extracted and analyzed as
described previously at the UCSD Lipidomics Core (78, 79). Briefly, sterols were extracted by dichloromethane/methanol (50:50; v/v), hydrolyzed, and separated using reverse-phase liquid chromatography using a 1.7 uM 2.1x150 mm Kinetex C18 column (Phenomenex) on an ACQUITY UPLC system (Waters) followed by analysis on QTRAP 6500 mass spectrometer (Ab Sciex). A mixture of deuterated standards (Avanti Polar Lipids) was used for internal standards. For intracellular cholesterol measurements, 1 x 10⁶ subconfluent astrocytes were pelleted and stored at -80°C. Cells were resuspended in PBS, mixed with chloroform:methanol (2:1 v/v), vortexed, and rotatated. Following centrifugation, the chloroform and lipid containing layer was transferred to a new tube, vacuum dried, and resuspended in Reaction Buffer E from the Amplex Red Cholesterol Assay (Invitrogen). Cholesterol was measured from the chloroform:methanol extracted samples using the Amplex Red Cholesterol Assay as directed by the manufacturer.

Cell Surface Biotinylation—For surface biotinylation of lipoprotein receptors, astrocytes were seeded in a 10 cm plate at a density of 2 x 10⁶ cells per plate in duplicate. After 48 hours, sub-confluent astrocytes were washed three times with ice cold PBS and then incubated with either PBS or 2 mM EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher) for 30 minutes at 4°C. Cells were washed three times with TBS pH 7.4 and lysed in RIPA buffer with protease and phosphatase inhibitors. For streptavidin pulldown, 250 µg protein lystate at 0.5 µg/µl was incubated with 100 µl of pre-washed PureProteome Streptavidin Magnetic Beads (EMD Millipore) by rotating overnight at 4°C. The next day, beads were immobilized and a sample of supernatant was saved to measure non-biotinylated intracellular protein. The beads were washed five times in cold PBS containing 1% Triton X-100 and biotinylated proteins were released from the streptavidin beads by boiling the samples in 2X NuPAGE LDS Sample Buffer (Invitrogen) at 100°C. Western blots were run with 5% of input, 5% of supernatant, and 50% of pull-down.

Preparation of Aβ Peptide—Lyophilized FITC labeled beta amyloid (1-42) (American Peptide) or Beta-Amyloid (1-42) HiLyte Fluor 647 (Anaspec) was solubilized following the manufacturer’s instructions using a minimal amount of alkaline 1.0% NH₄OH immediately followed by 1X PBS to a working concentration of 100 µM. Small aliquots were immediately stored at -80°C and only used once to eliminate variability due to freeze-thawing.

Flow Cytometry—For lipoprotein and bulk endocytosis assays, astrocytes were seeded in a 24-well plate at a density of 8 x 10⁴ cells per well. Two days later, cells were treated with 20 µg/ml BODIPY FL LDL or DiI LDL for one hour at 37°C, or 50 µg/ml Dextran-Fluorescein or Dextran-Alexa Fluor 647 (Life Technologies). Following incubation with labeled substrates, cells were washed with cold PBS, and dissociated with Trypsin (Invitrogen) for 5-10 minutes at 37°C to remove any ligand bound to the cell surface. For the transferrin recycling assay, astrocytes were seeded in a 24-well plate at a density of 8 x 10⁴ cells per well. Two days later, cells were treated with 100 µg/ml Transferrin-Alexa Fluor 647 (Life Technologies) for 10 minutes at 37°C. Following the 10 minute incubation, cells were washed with cold PBS and cold acid wash buffer to remove transferrin bound to the cell surface. Cells at the “0” time point were dissociated using Accutase, filtered, and stored on ice until analysis. The remaining conditions were “chased” with culture medium and harvested at the indicated time points. For analysis of cell surface LDLR protein, astrocytes were seeded in a 24-well plate at a density of 8 x 10⁴ cells per well. After 24 hours, astrocytes were treated with either DMSO or berberine (10 µg/mL, Selleck Chemicals) for 24 hours. Next, cells were washed with PBS and dissociated using an EDTA dissociation buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM Glucose, 5 mM KCl, 125 mM NaCl, and 2 mg/ml BSA) for 10 minutes at 37°C. Cells were then incubated with PE Mouse Anti-Human LDLR (BD Biosciences 565653) at a final concentration of 8 µg/ml on ice for 30 minutes. For continuous Aβ uptake assays, astrocytes were incubated with 500 nM FITC labeled beta amyloid (1-42) (American Peptide) or 500 nM Beta-Amyloid (1-42) HiLyte Fluor 647 (Anaspec). At the indicated time points, cells were harvested by
trypsinization to remove any surface-bound ligand and fixed with 4% paraformaldehyde (PFA) at room temperature for 15 minutes. Fixed cells were stored in PBS at 4°C until analysis. All experiments were analyzed on a BD Accuri C6 flow cytometer (BD Biosciences). 10,000 to 20,000 events were recorded per sample and the median fluorescence intensity was quantified.

**Immunofluorescence**—Astrocytes were seeded in 8-well chamber slides at a density of 3 × 10⁴ cells per chamber and fixed 2-3 days after plating. Briefly, cells were fixed with 4% PFA for 15 minutes at room temperature, permeabilized with 0.1% Triton X-100, and blocked in serum. The antibodies used for immunofluorescence experiments were anti-EQA1 (1:100; BD Biosciences 610456/7), anti-LRP1 (1:200; Abcam AB92544), and anti-M6PR (1:1,000, Abcam AB12894). Secondary antibodies were Alexa Fluor anti-mouse and anti-rabbit IgG (Invitrogen) and used at 1:200. Images were acquired on a Zeiss or Leica confocal microscope.

**Aβ and sAPP Measurements**—For secreted Aβ and sAPP measurements, astrocytes were seeded at 2.5 × 10⁵ cells per well of a 24-well plate. The following day, the medium was changed. After 5 days in culture, medium was harvested and run on a V-PLEX Aβ Peptide Panel 1 (6E10) Kit, sAPPα/sAPPβ Kit, and/or Swedish sAPPβ Kit (Meso Scale Discovery). For cellular Aβ measurements, 500 nM Aβ (1-42) (American Peptide) was supplemented to astrocyte culture medium. After 24 hours, cells were washed with PBS and fresh medium was added. At the indicated times, cell lysates were harvested using MSD Lysis Buffer with protease and phosphatase inhibitors and stored at -80°C until they were run on a V-PLEX Aβ Peptide Panel 1 (6E10) Kit (Meso Scale Discovery). These measurements were normalized to protein content using the Pierce BCA Protein Assay Kit (Thermo Fisher).

γ-Secretase inhibitor treatment was performed using 200 nM Compound E (EMD Chemicals) for 48 hours. β-Secretase inhibitor treatment was performed using 4 µM β-Secretase Inhibitor IV (Calbiochem) for 24 hours.

**Statistics**—All data was analyzed using GraphPad Prism Software. Statistical analysis comparing two groups was performed using Student’s t test. Statistical analysis comparing different genotypes to WT controls was performed by Dunnett’s multiple comparisons test. Data are depicted with bar graphs of the mean ± SD.
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**Conflict of Interest:** The authors declare that they have no conflicts of interest with the contents of this article.
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REFERENCES

1. Hardy, J. (1997) Amyloid, the presenilins and Alzheimer’s disease. *Trends Neurosci.* 20, 154–159
2. Holtzman, D. M., Morris, J. C., and Goate, A. M. (2011) Alzheimer’s disease: the challenge of the second century. *Sci. Transl. Med.* 3, 77sr1
3. Hardy, J., and Selkoe, D. J. (2002) The Amyloid Hypothesis of Alzheimer’s Disease: Progress and Problems on the Road to Therapeutics. *Science* (80-. ). 297, 353–357
4. Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L., and Pericak-Vance, M. A. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer’s disease in late onset families. *Science.* 261, 921–923
5. Liu, C.-C., Liu, C.-C., Kanekiyo, T., Xu, H., and Bu, G. (2013) Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. *Nat. Rev. Neurol.* 9, 106–18
6. Bertram, L., McQueen, M. B., Mullikin, K., Blacker, D., and Tanz, R. E. (2007) Systematic meta-analyses of Alzheimer disease genetic association studies: the AlzGene database. *Nat. Genet.* 39, 17–23
7. Jick, H., Zornberg, G. L., Jick, S. S., Seshadri, S., and Drachman, D. A. (2000) Statins and the risk of dementia. *Lancet.* 356, 1627–31
8. Yaffe, K., Barrett-Connor, E., Lin, F., and Grady, D. (2002) Serum lipoprotein levels, statin use, and cognitive function in older women. *Arch Neurol.* 59, 378–384
9. Rockwood, K., Kirkland, S., Hogan, D. B., MacKnight, C., Merry, H., Verreault, R., Wolfson, C., and McDowell, I. (2002) Use of lipid-lowering agents, indication bias, and the risk of dementia in community-dwelling elderly people. *Arch Neurol.* 59, 223–227
10. van der Kant, R., and Goldstein, L. S. B. (2015) Cellular Functions of the Amyloid Precursor Protein from Development to Dementia. *Dev. Cell.* 32, 502–515
11. Grimm, M. O. W., Grimm, H. S., Pätzold, A. J., Zinser, E. G., Halonen, R., Duering, M., Tschäpe, J. A., De Strooper, B., Müller, U., Shen, J., and Hartmann, T. (2005) Regulation of cholesterol and sphingomyelin metabolism by amyloid-beta and presenilin. *Nat. Cell Biol.* 7, 1118–23
12. Liu, Q., Zerbinatti, C. V, Zhang, J., Hoe, H.-S., Wang, B., Cole, S. L., Herz, J., Muglia, L., and Bu, G. (2007) Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1. *Neuron.* 56, 66–78
13. Tamboli, I. Y., Prager, K., Thal, D. R., Thelen, K. M., Dewachter, I., Pietrzik, C. U., St George-Hyslop, P., Sisodia, S. S., De Strooper, B., Heneka, M. T., Filippov, M. a, Müller, U., van Leuven, F., Lütjohann, D., and Walter, J. (2008) Loss of gamma-secretase function impairs endocytosis of lipoprotein particles and membrane cholesterol homeostasis. *J. Neurosci.* 28, 12097–12106
14. Pierrot, N., Tytca, D., D’auria, L., Dewachter, I., Gailly, P., Hendrickx, A., Tasiaux, B., Haylani, L. El, Muls, N., N’Kuli, F., Laquerrière, A., Demoulin, J. B., Campion, D., Brion, J. P., Courtoy, P. J., Kienlen-Campard, P., and Octave, J. N. (2013) Amyloid precursor protein controls cholesterol turnover needed for neuronal activity. *EMBO Mol. Med.* 5, 608–625
15. Wang, W., Mutka, A. L., Zmrljklak, U. P., Rozman, D., Tanila, H., Gylling, H., Remes, A. M., Huttunen, H. J., and Ikonom, E. (2014) Amyloid precursor protein α- and β-cleaved ectodomains exert opposing control of cholesterol homeostasis via SREBP2. *FASEB J.* 28, 849–860
16. Goldstein, J. L., DeBose-Boyd, R. A., and Brown, M. S. (2006) Protein sensors for membrane sterols. *Cell.* 124, 35–36
17. Deane, R., Wu, Z., Sagare, A., Davis, J., Du Yan, S., Hammad, K., Xu, F., Parisi, M., LaRue, B., Hu, H. W., Spijkers, P., Guo, H., Song, X., Lenting, P. J., Van Nostrand, W. E., and Zlokovic, B. V. (2004) LRP/amyloid β-peptide interaction mediates differential brain efflux of Aβ isoforms. *Neuron.* 43, 333–344
18. Zerbinatti, C. V, Wahrle, S. E., Kim, H., Cam, J. A., Bales, K., Paul, S. M., Holtzman, D. M., and Bu, G. (2006) Apolipoprotein E and low density lipoprotein receptor-related protein facilitate intraneuronal Aβ42 accumulation in amyloid model mice. *J. Biol. Chem.* 281, 36180–36186
19. Kim, J., Castellano, J. M., Jiang, H., Basak, J. M., Parsadanian, M., Pham, V., Mason, S. M., Paul,
S. M., and Holtzman, D. M. (2009) Overexpression of Low-Density Lipoprotein Receptor in the Brain Markedly Inhibits Amyloid Deposition and Increases Extracellular Aβ Clearance. Neuron. 64, 632–644
20. Basak, J. M., Verghese, P. B., Yoon, H., Kim, J., and Holtzman, D. M. (2012) Low-density lipoprotein receptor represents an apolipoprotein E-independent pathway of Aβ uptake and degradation by astrocytes. J. Biol. Chem. 287, 13959–13971
21. Liu, C.-C., Hu, J., Zhao, N., Wang, J., Na, W., Cirrito, J. R., Kanekiyo, T., Holtzman, D. M., and Bu, G. (2017) Astrocytic LRP1 Mediates Brain Aβ Clearance and Impacts Amyloid Deposition. J. Neurosci. 10.1523/JNEUROSCI.3442-16.2017
22. Nicolas, M., and Hassan, B. A. (2014) Amyloid precursor protein and neural development. Development. 141, 2543–2548
23. Yuan, S. H., Martin, J., Elia, J., Flippin, J., Paramban, R. I., Hefferan, M. P., Vidal, J. G., Mu, Y., Killian, R. L., Israel, M. A., Emre, N., Marsala, S., Marsala, M., Gage, F. H., Goldstein, L. S. B., and Carson, C. T. (2011) Cell-surface marker signatures for the Isolation of neural stem cells, glia and neurons derived from human pluripotent stem cells. PLoS One. 10.1371/journal.pone.0017540
24. Björkhem, I., Meaney, S., and Fogelman, A. M. (2004) Brain Cholesterol: Long Secret Life and Lowering Drug Working through a Unique Mechanism Distinct from Statins. J. Biol. Chem. 279, 39541–39548
25. Pfrieger, F. W., and Ungurer, N. (2011) Cholesterol metabolism in neurons and astrocytes. Prog. Lipid Res. 50, 357–371
26. Nieweg, K., Schaller, H., and Pfrieger, F. W. (2009) Marked differences in cholesterol synthesis between neurons and glial cells from postnatal rats. J. Neurochem. 109, 125–134
27. Brown, M. S., and Goldstein, J. L. (1997) The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell. 89, 331–340
28. Yang, C., McDonald, J. G., Patel, A., Zhang, Y., Umetani, M., Xu, F., Westover, E. J., Covey, D. F., Mangelsdorf, D. J., Cohen, J. C., and Hobbs, H. H. (2006) Sterol intermediates from cholesterol biosynthetic pathway as liver X receptor ligands. J. Biol. Chem. 281, 27816–27826
29. Zhang, Y., Sloan, S. A., Clarke, L. E., Caneda, C., Plaza, C. A., Blumenthal, P. D., Vogel, H., Steinberg, G. K., Edwards, M. S. B., Li, G., Duncan, J. A., Cheshier, S. H., Shuer, L. M., Chang, E. F., Grant, G. A., Gephart, M. G. H., and Barres, B. A. (2016) Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. Neuron. 89, 37–53
30. Llorente-Cortés, V., Costales, P., Bernués, J., Camino-Lopez, S., and Badimon, L. (2006) Sterol Regulatory Element-binding Protein-2 Negatively Regulates Low Density Lipoprotein Receptor-related Protein Transcription. J. Mol. Biol. 359, 950–960
31. Van Dam, E. M., Ten Broeke, T., Jansen, K., Spijkers, P., and Stoorvogel, W. (2002) Endocytosed transferrin receptors recycle via distinct dynamin and phosphatidylinositol 3-kinase-dependent pathways. J. Biol. Chem. 277, 48876–48883
32. Kong, W., Wei, J., Abidi, P., Lin, M., Inaba, S., Li, C., Wang, Y., Wang, Z., Si, S., Pan, H., Wang, S., Wu, J., Wang, Y., Li, Z., Liu, J., and Jiang, J.-D. (2004) Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. Nat. Med. 10, 1344–1351
33. Woodruff, G., Reyna, S. M., Dunlap, M., Van Der Kant, R., Callender, J. A., Young, J. E., Roberts, E. A., and Goldstein, L. S. B. (2016) Defective Transcytosis of APP and Lipoproteins in Human iPSC-Derived Neurons with Familial Alzheimer’s Disease Mutations. Cell Rep. 17, 759–773
34. Haass, C., Lemere, C. A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., and Selkoe, D. J. (1995) The Swedish mutation causes early-onset Alzheimer’s disease by beta-secretase cleavage within the secretory pathway. Nat. Med. 1, 1291–6
35. Games, D., Adams, D., Alessandri, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., and Gillespie, F. (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. Nature. 373, 523–7
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36. Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D. J. (1992) Mutation of the β-amyloid precursor protein in familial Alzheimer’s disease increases β-protein production. Nature. 360, 672–674

37. Cai, X. D., Golde, T. E., and Younkin, S. G. (1993) Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. Science (80-. ). 259, 514–516

38. Tamaoka, A., Odaka, A., Ishibashi, Y., Usami, M., Sahara, N., Suzuki, N., Nukina, N., Mizusawa, H., Shoji, S., and Kanazawa, I. (1994) APP717 missense mutation affects the ratio of amyloid beta protein species (A beta 1-42/43 and a beta 1-40) in familial Alzheimer’s disease brain. J. Biol. Chem. 269, 32721–4

39. Suzuki, N., Cheung, T. T., Cai, X. D., Odaka, A., Otvos, L., Eckman, C., Golde, T. E., and Younkin, S. G. (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science. 264, 1336–40

40. Chen, A. K., Cheng, Z., Behlke, M. A., and Tsourkas, A. (2008) Assessing the sensitivity of commercially available fluorophores to the intracellular environment. Anal. Chem. 80, 7437–7444

41. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. a, Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. a, Biere, a L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) Beta-secretase cleavage of Alzheimer’s amyloid protein precursor by the transmembrane aspartic protease BACE. Science. 286, 735–41

42. Choy, R. W.-Y., Cheng, Z., and Schekman, R. (2012) Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid β (Aβ) production in the trans-Golgi network. Proc. Natl. Acad. Sci. U. S. A. 109, E2077-82

43. Yamakawa, H., Yagishita, S., Futai, E., and Ishiura, S. (2010) Beta-Secretase inhibitor potency is decreased by aberrant beta-cleavage location of the “Swedish Mutant” amyloid precursor protein. J. Biol. Chem. 285, 1634–1642

44. Young-Pearse, T. L., Bai, J., Chang, R., Zheng, J. B., LoTurco, J. J., and Selkoe, D. J. (2007) A critical function for beta-amyloid precursor protein in neuronal migration revealed by in utero RNA interference. J. Neurosci. 27, 14459–14469

45. Kounnas, M. Z., Moir, R. D., Rebeck, G. W., Bush, A. I., Argraves, W. S., Tanzi, R. E., Hyman, B. T., and Strickland, D. K. (1995) LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted β-amyloid precursor protein and mediates its degradation. Cell. 82, 331–340

46. Knauer, M. F., Orlando, R. A., and Glabe, C. G. (1996) Cell surface APP751 forms complexes with protease nexin 2 ligands is internalized via the low density lipoprotein receptor-related protein (LRP). Brain Res. 740, 6–14

47. Pietrzik, C. U., Yoon, I.-S., Jaeger, S., Busse, T., Weggen, S., and Koo, E. H. (2004) FE65 Constitutes the Functional Link between the Low-Density Lipoprotein Receptor-Related Protein and the Amyloid Precursor Protein. J. Neurosci. 24, 4259–4265

48. Trommsdorff, M., Borg, J. P., Margolis, B., and Herz, J. (1998) Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. J. Biol. Chem. 273, 33556–33560

49. Bu, G., Cam, J., and Zerbinatti, C. (2006) LRP in amyloid-beta production and metabolism. Ann. N. Y. Acad. Sci. 1086, 35–53

50. Haß, S., Fresser, F., Köchl, S., Beyreuther, K., Utermann, G., and Baier, G. (1998) Physical interaction of ApoE with amyloid precursor protein independent of the amyloid Aβ region in vitro. J. Biol. Chem. 273, 13892–13897

51. Kamal, A., Stokin, G. B., Yang, Z., Xia, C.-H., and Goldstein, L. S. . (2000) Axonal Transport of Amyloid Precursor Protein Is Mediated by Direct Binding to the Kinesin Light Chain Subunit of Kinesin-I. Neuron. 28, 449–459

52. Kamal, A., Almenar-Queralt, A., LeBlanc, J. F., Roberts, E. A., and Goldstein, L. S. (2001) Kinesin-mediated axonal transport of a membrane compartment containing beta-secretase and presenilin-1 requires APP. Nature. 414, 643–648
53. Schmidt, V., Sporbert, A., Rohe, M., Reimer, T., Rehm, A., Andersen, O. M., and Willnow, T. E. (2007) SorLA/LR11 regulates processing of amyloid precursor protein via interaction with adaptors GGA and PACS-1. *J. Biol. Chem.* **282**, 32956–32964

54. Barrett, P. J., Song, Y., Van Horn, W. D., Hustedt, E. J., Schafer, J. M., Hadziselimovic, A., Beel, A. J., and Sanders, C. R. (2012) The Amyloid Precursor Protein Has a Flexible Transmembrane Domain and Binds Cholesterol. *Science* (80-. ). **336**, 1168–1171

55. Zheng, H., and Koo, E. (2011) Biology and pathophysiology of the amyloid precursor protein. *Mol. Neurodegener.* **6**, 27

56. Mauch, D. H., Nägler, K., Schumacher, S., Göritz, C., Müller, E. C., Otto, a, and Pfrieger, F. W. (2001) CNS synaptogenesis promoted by glia-derived cholesterol. *Science.* **294**, 1354–1357

57. Karch, C. M., Cruchaga, C., and Goate, A. M. (2014) Alzheimer’s disease genetics: From the bench to the clinic. *Neuron.* **83**, 11–26

58. Ryan, N. S., Nicholas, J. M., Weston, P. S. J., Liang, Y., Lashley, T., Guerreiro, R., Adamson, G., Kenny, J., Beck, J., Chavez-Gutierrez, L., de Strooper, B., Revesz, T., Holton, J., Mead, S., Rossor, M. N., and Fox, N. C. (2016) Clinical phenotype and genetic associations in autosomal dominant familial Alzheimer's disease: a case series. *Lancet Neurol.* **15**, 1326–1335

59. Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F., and Cole, G. (1996) Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. *Science* (80-. ). **274**, 99–102

60. Mucke, L., Masliah, E., Yu, G.-Q., Mallory, M., Rockenstein, E. M., Tatsuno, G., Hu, K., Koloddenko, D., Johnson-Wood, K., and McConlogue, L. (2000) High-Level Neuronal Expression of Aβ1–42 in Wild-Type Human Amyloid Protein Precursor Transgenic Mice: Synaptotoxicity without Plaque Formation. *J. Neurosci.* **20**, 4050–4058

61. Oddo, S., Caccamo, A., Shepherd, J. D., Murphy, M. P., Golde, T. E., Kayed, R., Metherate, R., Mattson, M. P., Akbari, Y., and LaFerla, F. M. (2003) Triple-transgenic model of Alzheimer’s Disease with plaques and tangles: Intracellular Aβ and synaptic dysfunction. *Neuron.* **39**, 409–421

62. Mawuenyega, K. G., Sigurdson, W., Ovod, V., Munsell, L., Kasten, T., Morris, J. C., Yarasheski, K. E., and Bateman, R. J. (2010) Decreased clearance of CNS beta-amyloid in Alzheimer’s disease. *Science.* **330**, 1774

63. Siman, R., Card, J. P., Nelson, R. B., and Davis, L. G. (1989) Expression of beta-amyloid precursor protein in reactive astrocytes following neuronal damage. *Neuron.* **3**, 275–285

64. Topper, R., Gehrmann, J., Banati, R., Schwarz, M., Block, F., Noth, J., and Kreutzberg, G. W. (1995) Rapid appearance of beta-amyloid precursor protein immunoreactivity in glial cells following excitotoxic brain injury. *Acta Neuropathol.(Berl).* **89**, 23–28

65. Almenar-Queralt, A. (2014) UV irradiation accelerates amyloid precursor protein (APP) processing and disrupts APP axonal transport. *J. Neurosci.* **34**, 3320–39

66. Pietrzik, C. U., Busse, T., Merriam, D. E., Weggen, S., and Koo, E. H. (2002) The cytoplasmic domain of the LDL receptor-related protein regulates multiple steps in APP processing. *EMBO J.* **21**, 5691–5700

67. Ulery, P. G., Beers, J., Mikhailenko, I., Tanzi, R. E., Rebeck, G. W., Hyman, B. T., and Strickland, D. K. (2000) Modulation of beta-amyloid precursor protein processing by the low density lipoprotein receptor-related protein (LRP). Evidence that LRP contributes to the pathogenesis of Alzheimer’s disease. *J. Biol. Chem.* **275**, 7410–7415

68. Kang, D. E., Pietrzik, C. U., Baum, L., Chevallier, N., Merriam, D. E., Kounnas, M. Z., Wagner, S. L., Troncoso, J. C., Kawas, C. H., Katzman, R., and Koo, E. H. (2000) Modulation of amyloid β-protein clearance and Alzheimer’s disease susceptibility by the LDL-receptor-related protein pathway. *J. Clin. Invest.* **106**, 1159–1166

69. Marzolo, M. P., and Bu, G. (2009) Lipoprotein receptors and cholesterol in APP trafficking and proteolytic processing, implications for Alzheimer’s disease. *Semin. Cell Dev. Biol.* **20**, 191–200

70. Israel, M. A., Yuan, S. H., Bardy, C., Reyna, S. M., Mu, Y., Herrera, C., Heffernan, M. P., Van Gorp, S., Nazor, K. L., Boscolo, F. S., Carson, C. T., Laurent, L. C., Marsala, M., Gage, F. H.,
Astrocytic APP Controls LDL Receptor Function

Remes, A. M., Koo, E. H., and Goldstein, L. S. B. (2012) Probing sporadic and familial Alzheimer’s disease using induced pluripotent stem cells. Nature. 482, 216–220

Woodruff, G., Young, J. E., Martinez, F. J., Buen, F., Gore, A., Kinaga, J., Li, Z., Yuan, S. H., Zhang, K., and Goldstein, L. S. B. (2013) The Presenilin-1 dE9 Mutation Results in Reduced γ-Secretease Activity, but Not Total Loss of PS1 Function, in Isogenic Human Stem Cells. Cell Rep. 5, 974–985

Young, J. E., Boulanger-Weill, J., Williams, D. A., Woodruff, G., Buen, F., Revilla, A. C., Herrera, C., Israel, M. A., Yuan, S. H., Edland, S. D., and Goldstein, L. S. B. (2015) Elucidating molecular phenotypes caused by the SORL1 Alzheimer’s disease genetic risk factor using human induced pluripotent stem cells. Cell Stem Cell. 16, 373–385

Gore, A., Li, Z., Fung, H.-L., Young, J. E., Agarwal, S., Antosiewicz-Bourget, J., Canto, I., Giorgetti, A., Israel, M. A., Kiskinis, E., Lee, J.-H., Loh, Y.-H., Manos, P. D., Montserrat, N., Panopoulos, A. D., Ruiz, S., Wilbert, M. L., Yu, J., Kirkness, E. F., Izpisua Belmonte, J. C., Rossi, D. J., Thomson, J. A., Eggen, K., Daley, G. Q., Goldstein, L. S. B., and Zhang, K. (2011) Somatic coding mutations in human induced pluripotent stem cells. Nature. 471, 63–7

Levy, S., Sutton, G., Ng, P. C., Feuk, L., Halpern, A. L., Walenz, B. P., Axelrod, N., Huang, J., Kirkness, E. F., Denisov, G., Lin, Y., MacDonald, J. R., Pang, A. W. C., Shago, M., Stockwell, T. B., Tsiamouris, A., Bafna, V., Bansal, V., Kravitz, S. A., Busam, D. A., Beeson, K. Y., McIntosh, T. C., Remington, K. A., Abril, J. F., Gill, J., Borman, J., Rogers, Y. H., Frazier, M. E., Scherer, S. W., Strausberg, R. L., and Venter, J. C. (2007) The diploid genome sequence of an individual human. PLoS Biol. 5, 2113–2144

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., and Church, G. M. (2013) RNA-guided human genome engineering via Cas9. Science (80-. ). 339, 823–826

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., and Zhang, F. (2013) Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308

D’Antonio, M., Woodruff, G., Nathanson, J. L., D’Antonio-Chronowska, A., Arias, A., Matsui, H., Williams, R., Herrera, C., Keyna, S. M., Yeo, G. W., Goldstein, L. S. B., Panopoulos, A. D., and Frazer, K. A. (2017) High-Throughput and Cost-Effective Characterization of Induced Pluripotent Stem Cells. Stem Cell Reports. 8, 1101–1111

Quéhenberger, O., Armando, A. M., Brown, A. H., Milne, S. B., Myers, D. S., Merrill, A. H., Bandyopadhyay, S., Jones, K. N., Kelly, S., Shaner, R. L., Sullards, C. M., Wang, E., Murphy, R. C., Barkley, R. M., Leiker, T. J., Raetz, C. R. H., Guan, Z., Laird, G. M., Six, D. a, Russell, D. W., McDonald, J. G., Subramaniam, S., Fahy, E., and Dennis, E. a (2010) Lipidomics reveals a remarkable diversity of lipids in human plasma. J. Lipid Res. 51, 3299–3305

McDonald, J. G., Smith, D. D., Stiles, A. R., and Russell, D. W. (2012) A comprehensive method for extraction and quantitative analysis of sterols and secosteroids from human plasma. J. Lipid Res. 53, 1399–1409
The abbreviations used are: AD, Alzheimer’s disease; APP, amyloid precursor protein; Aβ, amyloid-beta; KO, knockout; hiPSC, human induced pluripotent stem cell; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LRP1, low density lipoprotein receptor-related protein; SREBP, sterol-regulatory element-binding protein.
FIGURE 1: Generation of APP-KO hiPSCs using CRISPR/Cas9-targeted genome editing

(A) Diagram of CRISPR/Cas9 workflow to generate isogenic hiPSCs (B) Summary diagram of the validation of isogenic APP-KO hiPSCs, astrocytes, and neurons.
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FIGURE 2: Characterization of APP-KO isogenic cells

(A) Insertions or deletions induce premature stop codon formation in both alleles of two independently derived APP-KO hiPSC clones, IA1 and IB6. (B) qRT-PCR analysis of APP, APLP1, and APLP2 mRNA levels in NPCs (n \( \geq 8 \) from three independent experiments), (C) purified neurons (n \( \geq 8 \) from two independent experiments), and (D) astrocytes (n \( \geq 6 \) from at least two independent experiments), shows little detectable APP mRNA (p < 0.0001) and no significant differences in APLP1 or APLP2 mRNA levels in neurons or astrocytes. There was a significant increase in APLP1 (p = 0.0082) and APLP2 (p = 0.0035) mRNA in NPCs. All qRT-PCR data was normalized to RPL13A, RPL27, and TBP. (E) Representative western blots from WT and APP-KO NPCs using antibodies for APP and APP family members, APLP1 and APLP2, shows no detectable APP protein, but a slight elevation in APLP1 and APLP2. (F) The percentage of neurons identified by flow cytometry using a CD44+/CD184+/CD24+ cell...
surface signature after three weeks of neural differentiation shows that loss of APP does not affect neuronal differentiation (n ≥ 7 from four independent experiments). (G) The percentage of astrocytes positive for CD44, CD184, and GFAP by flow cytometry is not different between WT and APP-KO astrocytes (n = 12 from three independent experiments). Data are depicted with bar graphs of the mean ± SD.
FIGURE 3: APP-KO astrocytes have altered cholesterol metabolism
(A) Lipidomics mass spectrometry quantification of the cholesterol precursors: desmosterol (**p = 0.0025, ***p = 0.0003; n ≥ 4) and (B) 7-dehydrocholesterol (p < 0.0001; n ≥ 4) in astrocytes, neurons, and NPCs show much higher levels of cholesterol precursors in astrocytes. (C) Quantification of total cellular cholesterol levels by Amplex Red Cholesterol Assay Kit show decreased cholesterol in APP-KO astrocytes (p = 0.0226; n ≥ 13 from three independent experiments). (D) qRT-PCR analysis of mRNA levels from astrocytes grown in medium containing 3% FBS show upregulated HMGCR (p = 0.0382), LDLR (p = 0.0427), and LRP1 (p = 0.0157) gene expression in APP-KO astrocytes (n ≥ 10 from at least two independent experiments). (E) qRT-PCR analysis of HMGCR, LDLR, and LRP1 mRNA levels from astrocytes grown with lipoprotein-depleted serum (LDS) show no significant differences in expression between WT and APP-KO astrocytes (n ≥ 8 from at least two independent experiments). (F) Quantification of mRNA induction of HMGCR, LDLR, and LRP1 mRNA by qRT-PCR 24 hours after changing growth medium from 3% FBS containing medium to lipoprotein-depleted medium shows no significant differences in the fold induction of SREBP-target genes in WT and APP-KO astrocytes (n ≥ 4 from at least two independent experiments). Data are depicted with bar graphs of the mean ± SD.
FIGURE 4: APP-KO astrocytes have impaired lipoprotein endocytosis
(A) Quantification of LDL endocytosis by flow cytometry shows reduced lipoprotein endocytosis in APP-KO astrocytes (p = 0.0001; n = 24 from four independent experiments). (B) Quantification of bulk endocytosis of dextran by flow cytometry show no significant differences between WT and APP-KO astrocytes (n = 24 from four independent experiments). (C) Quantification of transferrin recycling over time by flow cytometry shows no difference in the rate of recycling between WT and APP-KO astrocytes (n ≥ 10 from three independent experiments). (D) Quantification of cell-surface LDLR protein of WT and APP-KO astrocytes upon DMSO or berberine (BBR)-treatment by flow cytometry. While BBR-treatment
did upregulate cell surface LDLR (p < 0.0001), there were no significant differences between WT and APP astrocytes (n ≥ 2 from two independent experiments). (G) Cell surface biotinylation and western blot analysis of biotinylated LRP1 or (H) LDLR at the cell surface (50% pull-down), quantified in (E) and (F) in WT or APP-KO astrocytes shows no significant differences in surface receptor levels between WT and APP-KO astrocytes (n = 6 from three independent experiments). (I-L) Western blot analysis of 5% input LRP1 or LDLR show elevated total LRP1 (p = 0.0013) and LDLR (p = 0.0013) protein levels in APP-KO astrocytes (n = 12 from three independent experiments). (M-P) Western blot analysis and quantification of 5% supernatant or intracellular lipoprotein receptor levels showed elevated intracellular LRP1 (p = 0.0452) and LDLR (p = 0.0083; n = 12 from three independent experiments). (Q) Immunofluorescence images of enlarged LRP1 puncta in APP-KO astrocytes compared to WT (p = 0.0050; n ≥ 19 from two independent experiments). Data are depicted with bar graphs of the mean ± SD.
FIGURE 5: FAD astrocytes exhibit alterations in APP processing
(A-C) FL APP (22C11), APP CTF, and actin protein levels in astrocytes by Western Blot. (B) Quantification of FL APP protein normalized to actin shows a 30% reduction in FL APP in APP\textsuperscript{Swe/Swe} astrocytes (**p = 0.0007; n ≥ 3 from at least three independent experiments). (C) Quantification of APP α-CTF protein in astrocytes normalized to total CTF protein after 48 hours of 200 nM Compound E treatment (*p = 0.0306; n ≥ 3 from at least three independent experiments). (D) Quantification of APP β-CTF protein in astrocytes normalized to total CTF protein after 48 hours of 200 nM Compound E treatment (**p = 0.0012, ****p < 0.0001; n ≥ 3 from at least three independent experiments). (E) Quantification of the Aβ42/Aβ40 ratio after measuring secreted Aβ peptides from astrocytes by MSD immunoassay (****p < 0.0001; n ≥ 6 from three independent experiments). (F) Secreted Aβ40 from
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... astrocytes (****p < 0.0001; n ≥ 6 from three independent experiments). (G) Secreted Aβ42 from astrocytes (****p < 0.0001, **p = 0.0012; n ≥ 6 from three independent experiments). (H) Secreted Aβ38 from astrocytes (****p < 0.0001; n ≥ 6 from three independent experiments). (I) Secreted sAPPα from astrocytes (*p = 0.0362, ***p < 0.0002; n ≥ 5 from three independent experiments). (J) Secreted sAPPβ from astrocytes (**** p < 0.0001; n ≥ 5 from three independent experiments). (K) Secreted Swedish sAPPβ from astrocytes (**p = 0.0052; n = 8 from two independent experiments). Data are depicted with bar graphs of the mean ± SD.
FIGURE 6: APP<sup>Swe/Swe</sup> astrocytes recapitulate APP-KO phenotypes

(A) Quantification of LDL endocytosis in FAD astrocytes by flow cytometry demonstrates that APP<sup>Swe/Swe</sup> astrocytes also exhibit reduced lipoprotein endocytosis (p = 0.0242; n ≥ 4 from three independent experiments).

(B) Quantification of bulk endocytosis in FAD astrocytes by flow cytometry for fluorescently-tagged dextran shows no significant differences between WT and all genotypes (n ≥ 6...
from three independent experiments). (C) Western blot analysis of full length SREBP1 protein, cleaved or mature SREBP1 protein, LDLR, and actin show increased levels of (D) FL SREBP1 protein (**p = 0.0071, ***p = 0.0004; n ≥ 4 from four independent experiments), (E) cleaved SREBP1 protein (**p = 0.0077, ***p = 0.0005; n ≥ 4 from four independent experiments), and an (F) increased ratio of cleaved/FL SREBP1 protein (*p = 0.0204, **p = 0.0052; n ≥ 4 from four independent experiments) in APP-KO and APP\textsuperscript{Swe/Swe} astrocytes. (G) APP-KO and APP\textsuperscript{Swe/Swe} astrocytes also demonstrate increased total LDLR protein (*p = 0.0326, **p = 0.0022; n ≥ 5 from five independent experiments). Data are depicted with bar graphs of the mean ± SD.
FIGURE 7: APP-KO and APP\textsuperscript{Swe/Swe} astrocytes have impaired Aβ internalization

(A) Representative immunofluorescence images of WT astrocytes treated with Aβ-FITC for 15 minutes, harvested at different time points, and stained for EEA1, M6PR, and Aβ. (B) Quantification of puncta count shows relatively equal numbers of EEA1 and M6PR over time, but decreased Aβ-FITC puncta in...
WT astrocytes over 72 hours. (C) Colocalization analysis shows that the percent of Aβ with M6PR increased over time, but with a slight reduction from 48 to 72 hours. Data are representative of two independent experiments. (D) Flow cytometry analysis of continuous Aβ internalization in WT astrocytes treated with Aβ-FITC or Aβ-HiLyte Fluor 647 shows increased Aβ over the course of 48 hours, but a reduction of intracellular Aβ between 48 and 72 hours in astrocytes treated with the pH-sensitive Aβ-FITC, but not pH-insensitive Aβ-HiLyte Fluor 647 (n ≥ 10 from two independent experiments). (E) Quantification of cellular Aβ42 by MSD immunoassay in WT astrocytes over time after pre-treatment with Aβ42 for 24 hours (n = 6 from two independent experiments). (F) Quantification of Aβ internalization in APP-KO and FAD astrocytes by flow cytometry demonstrates that APP-KO (p < 0.0001) and APP<sup>Swe/Swe</sup> (p = 0.0012) astrocytes have impaired Aβ uptake (n ≥ 6 from two independent experiments). Data are depicted with bar graphs of the mean ± SD.
**FIGURE 8: Pharmacological inhibition of β-secretase reverses LDL and Aβ endocytosis defects in APP<sup>Swe/Swe</sup> astrocytes**

(A & B) Quantification of cell surface biotinylation experiments show that APP<sup>Swe/Swe</sup> astrocytes have reduced cell surface APP levels, identified in the pull-down (PD) lanes, compared to WT astrocytes (p = 0.0016). Western blots were run with 5% of input, 5% of supernatant (sup.), and 50% of pull-down (n ≥ 5 from three independent experiments). (C) Secreted Aβ40 peptide levels, measured by MSD immunoassay, in WT and APP<sup>Swe/Swe</sup> astrocytes after 24 hour-treatment ± a β-secretase inhibitor (BSI), exhibit significant reductions in secreted Aβ40 peptides upon BSI-treatment (*p = 0.0154, **p = 0.0020; n = 6 from two independent experiments). (D) Flow cytometry analysis of LDL (n ≥ 4 from two independent experiments) and (E) Aβ endocytosis (n ≥ 4 from two independent experiments) in WT and APP<sup>Swe/Swe</sup> astrocytes ± BSI-treatment shows that β-secretase inhibition reverses defects in APP<sup>Swe/Swe</sup> (LDL p = 0.0120, Aβ p = 0.0092), but not APP-KO astrocytes. Data are depicted with bar graphs of the mean ± SD.